

12 June 1980

Nuclear proliferation and World War III

What with Iran, Afghanistan and southern Africa, most people agree that these are dangerous times. For that reason it is all the more important that there should be a cool appraisal of where the dangers lie. Unhappily, too many of us are too often tempted to look for technical solutions to what are essentially political problems. So much is apparent from the otherwise fine declarations on arms control with which Mr James Callaghan, still leader of the British Labour Party, sought to elevate the discussions of his party's special conference on 31 May. China, said Mr Callaghan, on the strength of a recent visit, is convinced that sooner or later, and probably within the next twenty-five years, there will be a major conflict between itself and the Soviet Union. In preparation, China is working hard on the development of long-range missiles and the warheads they would carry. At the same time, familiarity with nuclear technology is spreading. So efforts must be redoubled to negotiate a comprehensive test-ban, to make a start on Salt III and to do something further to inhibit the spread of nuclear weapons. Few will quarrel with the conclusions, but many will quite properly ask whether Mr Callaghan's chilling scenario of the coming quarter of a century would be substantially modified even if the current arms control agenda were miraculously to be completed overnight (which it will not be).

The trouble is that the relationship between arms control treaties and the prospects for World War III are by no means as straightforward as Mr Callaghan suggested. If, indeed, his Chinese hosts are right in their estimation of the prospects ahead of them, they will most probably simply be confirmed in their determination to stand aside from the existing instruments of arms control — the Partial Test-Ban Treaty and the Non-Proliferation Treaty in particular. If, with the passage of time, that determination persists, one consequence may be that other parties to these treaties, and the existing nuclear powers in particular, will give up their own adherence on the grounds that continued membership would put them at a disadvantage with respect to China. In short, arms control treaties in themselves are neither a sufficient nor even a necessary condition for the avoidance of World War III. Rather, progress in the negotiation of arms control treaties is a sign of governments' willingness to accommodate inevitable political tensions within the conventional framework of international relations. So much should be apparent from the way in which, in the past six months, Salt II has languished in the United States Congress and the Soviet Union has declined the American invitation to talk about tactical nuclear weapons (the intended core of Salt III).

Fortunately it does not follow that arms control treaties are merely symbolic. The Antarctic Treaty is a good illustration. No government's interest would be served by an international competition to build bigger and better military installations in this strategically irrelevant part of the world, but many of them would feel impelled to follow suit if their rivals began. The treaty serves the mutual interest of even quite strongly conflicting parties. It represents the willingness in the early 1960s of three out of the (now) five nuclear powers to give up testing nuclear weapons in the atmosphere. No doubt it has been more than a mere inconvenience for each of them, even though continued testing underground has plainly made possible new ranges of warheads. Others have benefited from the decrease of radioactive fall-out.

The Comprehensive Test-Ban Treaty would be a much more significant brake on the development of new warheads in the United States and the Soviet Union (for which reason it is ironical that the present hiatus in the negotiations centres on the British refusal to base ten monitoring stations on British territory). But a successful treaty could not last for long if it did not include China. To expect otherwise is to wish for the moon.

The benefits of the Non-Proliferation Treaty (NPT) are different again, and harder to define. Much will (or should) be heard of them in the next few months, in the run-up to the Second Review Conference due to open in Geneva on 11 August. Ostensibly, the treaty serves Mr Callaghan's goal of limiting the spread of nuclear weapons — or at least of restricting them to the existing nuclear powers. Non-nuclear powers are required to forswear nuclear arms and to accept international control and inspection provided by the International Atomic Energy Agency. Nuclear powers can keep their weapons but must work towards further measures of strategic disarmament and also help with the development of civil nuclear technology in non-nuclear states. For one reason and another, however, there has been a subtle but important change in the function of the treaty since it was negotiated in the late 1960s. It is far from clear what will happen in Geneva later in the summer.

The nuclear powers can, however, expect to be hauled over the coals (as they were at the first review conference five years ago) for backsliding on their undertaking to negotiate substantial measures of strategic disarmament. The plight of Salt II will be a black mark. So will be the hiatus in the Comprehensive Test-Ban Treaty. The nuclear powers may however escape more lightly than they feared, if only because their critics will be divided on where to put the blame. It would be a brash non-nuclear power that would in present circumstances expect that substantial agreements on strategic weapons could be made to stick.

There will be more fierce argument about the second of the undertakings originally given by the nuclear powers — that they would assist the free development of civil nuclear power elsewhere. Originally this provision was designed to sweeten the pill of the frankly discriminatory nature of the treaty. There have been two developments in the past five years which stick in the throats of the non-nuclear powers. First, a number of governments which are potential suppliers of nuclear equipment (including France, which is not a signatory of the treaty) have worked out common rules for the supply of reactors, diffusion plants and the like. Second, the United States Administration three years ago promulgated tighter rules to govern the supply and use of nuclear fuel which, by being unilateral and outside the framework of the NPT, have further injured the pride of the non-nuclear signatories (often cajoled to sign by the United States), already smarting from the sense that the NPT is unfairly discriminatory as between nuclear and non-nuclear powers. Why, some non-nuclear powers ask, if we have signed the treaty and agreed to obey the rules, should the governments that promised to help us impose still further restrictions?

Especially now that the International Fuel Cycle Evaluation Study has shown that the dangers of the diversion of plutonium to military purposes cannot be prevented by requiring that everybody should build some diversion-proof reactor, it would be seemly if the United States government were to subsume its rules



for the supply of nuclear fuel within the NPT. (It would also be sensible if the Administration were to permit again the reprocessing of nuclear fuel within the United States, if only to empty some of the reactor cooling ponds now stacked with spent uranium; the chances are, however, that that will not be possible until after the presidential election in November.) If the nuclear powers earnestly wish to use the NPT as a means of inhibiting the spread of nuclear weapons, they should see that it is the chief instrument of their common policy.

How effective would be such a strengthened treaty? The record of the past ten years is mildly encouraging. The number of signatories has grown to 120 (but states such as India and Pakistan, Israel and Egypt, France and China conspicuously remain outside). Signature of the treaty has become more and more a precondition for buying nuclear equipment and fuel (which is why it is unfortunate that the Soviet Union is proposing to supply a nuclear reactor to Cuba, a determined non-signatory). Technically, the safeguards system is working more smoothly than five years ago — and would be even more effective if the treaty required regular (say annual) publication of the inputs to

and the outputs from national fuel cycles. The absence of France from the list of signatories is not as serious as it seems (and seemed ten years ago), for the French government has openly acknowledged that it will behave as if it were a nuclear power and member of the treaty. The absence of China is more worrying. Would it not be sensible that the first three nuclear powers should swallow their pride and agree that the definition of what constitutes a nuclear power should be broadened so as to include France and China?

Unfortunately, none of this will serve Mr Callaghan's wider purpose of postponing World War III. The most immediate danger is not that states which do not have nuclear weapons will acquire them but that states that have them will use them. Current arms control treaties, valuable though they are, have no immediate bearing on the likelihood that nuclear powers will use their nuclear weapons. Treaties in prospect, Salt II and III especially, would limit the chance of some kinds of conflicts but leave others unrestrained. As always, the immediate steps to be taken in postponement of World War III are political and not technical.

Foods, Fads and the National Academy

The National Academy of Sciences in the United States should have known it was stirring a hornet's nest by publishing last week a document unforgivably called "Toward Healthful Diets", prepared by the Food and Nutrition Board of the National Research Council, the academy's policy-pronouncing arm. For the document dares to cast doubt on a belief now as firmly held and cherished in the United States as was the concept of motherhood in the 1950s (before everything changed) — the belief that cholesterol and saturated fats in the human diet are akin to poisons. American newspapers have been up in arms. Luckless members of the committee responsible for the document have been quizzed about their earnings as consultants for the food industry, three of them owning up to deriving up to ten per cent of their incomes from this source. On the other hand, the trade associations representing the egg and dairy industries have sprung to the Food and Nutrition Board's defence with such alacrity that its members must be wondering whether, with friends like that, they have need of enemies.

In retrospect, the Food and Nutrition Board will also acknowledge that its document was a tactless publication. Over the years the board, originally set up in 1941, has acquired a useful reputation, largely on the strength of its recommendations on the nutritional composition of the American diet. It is, for example, the source of the influential "Recommended Dietary Allowances" for the various components of the human diet. Last week's document is however not so much a measured report of scientific deliberations as an opinion. Somewhere in the cumbersome procedure within the National Academy for reviewing the publications of the National Research Council, somebody should have recognised that publication would cause a storm, if only a storm in a teacup. It is also likely that the board's objective, "to reduce the confusion in the mind of the public" stemming from the flood of contradictory recommendations on diet in the United States, would have been better served if its conclusions had been bolstered by more careful argument.

The board's impatience is, however, understandable. The past few years have indeed been marked by an over-excited flood of advice to the general public about the proper management of the human diet. Some of it has come from official sources in the United States. Some of it has been contradictory. Collectively, and by its sheer volume, this advice must have served to give at least some members of the American public the impression that eating as such is dangerous. Others have been persuaded to an over-zealous avoidance of this or that component of diet without a sufficient regard for the biological complexity of the bodies they are seeking to nourish. The argument about the role of saturated fatty acids, and of cholesterol, as contributors to cardiovascular

disease goes back twenty years — and cannot now be said to have been resolved. But the Food and Nutrition Board is also concerned, in its latest document, with the dietary habits which are evolving or being recommended for the avoidance of other degenerative diseases — obesity, hypertension, some forms of cancer and diabetes mellitus. The theme running through its opinion is that diet may indeed influence long-term health, but that it is hardly ever possible to pin down exact relationships, valid for a whole population, between the amount of some component in the diet and the risk of disease in later life. What nutritionist would quarrel with that?

The board's account of the relationship between diet and the risk of cardiovascular disease is more moderate than its critics have supposed. The board accepts that high serum concentrations of cholesterol are associated with a high risk of cardiovascular disease. It points out, however, that the three somewhat arbitrarily distinguished classes of lipoproteins in the blood appear to be differently associated with later risk. Plainly there is an urgent need for a better understanding of the underlying biochemical processes. In the meantime, the board is entitled to its opinion that it remains to be demonstrated that high concentrations of serum cholesterol are more than mere symptoms of a predisposition to cardiovascular disease. The board also points out that the presence of cholesterol in the diet is not linearly related to the concentration of cholesterol in the serum: the more cholesterol in the diet, the less efficiently it is absorbed. And natural metabolic processes account for twice as much cholesterol as that in the average diet. The board does however acknowledge, as it must, that less saturated fatty acid in the diet means less cholesterol in the blood. This, too, is beyond dispute.

The board's advice, based on this analysis and a brief consideration of the epidemiological studies which have been carried out in recent years, is that there can be no uniform rule, applying to a whole population, for regulating either the ratio of saturated to unsaturated fats in the human diet or the amount of cholesterol which it is safe to eat. Quite properly, the board says that people who lead active lives — manual workers, for example — may need to include more natural fat in their diet than other people. All this is sensible enough, and it is possible that the row which has burst about the board's head may give pause to the organisations which have been advocating the adoption of specific values for the amounts of saturated fats and of cholesterol in the diet; they have often seemed unaware that they have been playing into the hands of faddism. The board itself, however, would be well advised also to think again, and recognise that the tide of faddism will be turned back only by a much more persuasive document.

An outbreak of piracy in the literature

A rash of what appears to be piracy has turned up in the scientific literature. At least three cases are known in which either authors or editors of journals have drawn attention to the appearance elsewhere of articles which are substantially identical with articles that they have published. A common theme in these events is the appearance of the name of Dr E.A.K. Alsabti as one of the authors of the pirated articles. One illustration of the phenomenon is the appearance last year in the *Japanese Journal of Medical Science and Biology* of an article "Effect of platinum compounds on murine lymphocyte mitogenesis" which inspection shows to be a close copy of an article by Dr D. Wierda and Dr T.L. Pazdernick also published last year in the *European Journal of Cancer*. Among the three authors of the article in the Japanese journal is E.A.K. Alsabti, whose address is given as the Royal Scientific Society, Amman, Jordan.

Platinum compounds in cancer

The objectives of the research reported by Wierda and Pazdernick, and in the paper by Alsabti *et al.*, are given as the investigation of the effects of various platinum compounds of the spleen lymphocytes known as T and B cells. The issue is of practical importance because platinum compounds, like other anti-tumour drugs, kill not merely tumour cells but also the circulating lymphocytes of the immune system.

Interest in platinum compounds dates back more than a decade, when the compound DDP (*cis*-dichlorodiamine platinum) was shown by Rosenberg *et al.* (*Nature*, 222, 385; 1969) to be effective against tumours in mice. Daniel Wierda says that his own interest in the field dates back to 1975, when his then supervisor, Dr Pazdernick, at the Department of Pharmacology of the University of Kansas, suggested a library search for evidence on which to base a search for analogues of DDP whose effects on the immune system would be less marked than those of the original compound.

In the research described in the two papers now published, mice have been used to demonstrate that the analogues of DDP are more toxic to T than to B cells. From this emerges the suggestion that platinum compounds might be used in conjunction with drugs which are more toxic to B cells than to T cells. Therapeutic regimes using DDP have been introduced in the past few years, and both papers come to the conclusion that clinical trials with the analogues now studied should be carried out.

Attempts in the past few days to trace Dr Alsabti and his fellow authors have been unsuccessful. The Scientific Attaché at the Jordanian Embassy in London said on the telephone on Monday this week that he had heard of Dr Alsabti, but that he knew nothing of his present whereabouts, believing him to be in Jordan.

The attaché also said that Dr Alsabti had no present affiliation with the Royal Scientific Society in Jordan. "Some years ago", Dr Alsabti had been allowed to use the address of the Royal Scientific Society, but this arrangement had since been terminated. He had no knowledge of the two other authors.

One feature of the paper in the Japanese journal is that readers are invited to send requests for reprints to an address in Brighton, "c/o Mrs W. Aljaff, Flat No. 5, 8 Norfolk Terrace, Brighton BN1 3AD, England". No name resembling Aljaff appears in the current Brighton telephone directory. A caller at 8 Norfolk Terrace earlier this week discovered that the occupant of Flat No. 5 spoke with a pronounced English accent and that he did not know of either Aljaff or Alsabti.

The two versions of the paper differ from each other in minor but perhaps significant details. The title of the paper in the Japanese journal is given as "Effect of platinum compounds on murine lymphocyte mitogenesis". The two American authors acknowledge the help of their technical assistant, while the three Jordanian authors say that "This work was supported by His Royal Highness Crown Prince Hassan of Jordan".

The references quoted at the end of Jordanian version do not however include the reference to earlier work of Pazdernick, one of the two American authors whose joint paper with Dr Wierda in the *European Journal of Cancer* had referred to a paper by Pazdernick first published in 1978.

The dates at which the two manuscripts were processed by the respective journals

are very similar. Dr H. J. Tagnon, editor of the *European Journal of Cancer*, has confirmed in writing that the American paper was received in the Brussels office on 10 October 1978 and accepted for publication on 5 January 1979. The version published in the Japanese journal carries a note "Received November 20, 1978", implying that the manuscript had been received in Tokyo before a final decision on the American paper had been made in Brussels.

The editor of the Japanese journal during the period concerned, Dr Hideo Fukumi, said in Tokyo on Monday this week that he had now retired from that post and did not know who had succeeded him. He said that he could remember nothing of the circumstances attending the publication of the paper by Alsabti *et al.*

The processing of the corresponding American paper in Brussels appears to have followed a more or less normal course. It was sent to two referees in the United States, one of whom replied (returning the manuscript and the prints of the diagrams eventually published with the article). After some delay, it turned out that the second referee had died, and that copy of the manuscript has not since been recovered.

Dr Wierda says that he is sure in his own mind that no unauthorized person would have been able to obtain a copy of his material in advance of publication, but Dr Tagnon is equally firm in his assurances that there could have been no unauthorized leakage from his office.

Whatever the circumstances, this appears not to be the first occasion on which Dr Alsabti has had the misfortune to be involved with issues of copyright to written materials.

In April this year, Professor E Frederick Wheelock of the Jefferson Medical College in Philadelphia wrote to *The Lancet* to complain that a section of a grant proposal which he had written for the US Public Health Service (and which was sub-

Two conclusions — Alsabti *et al.* superimposed

In conclusion, we have observed that the platinum complexes tested were more toxic to splenic T lymphocyte function than splenic B lymphocyte function. Collectively, the results of these experiments demonstrated that the type of immunosuppression produced by the platinum compounds is markedly different from the type of inhibition caused by such commonly used immunosuppressants as cyclophosphamide (Turk and Poulter, 1972; Gale *et al.*, 1975). Because of this unique suppression, we believe that further clinical testing of these compounds as potential antitumor agents is warranted.

In conclusion, we have observed that the platinum complexes tested were more toxic to splenic T lymphocyte function than splenic B lymphocyte function. We have also demonstrated that DBCH and DBCP were more toxic to splenic T lymphocyte function than splenic B lymphocyte function. Collectively, the results of these experiments demonstrated that the type of immunosuppression produced by the platinum compounds is markedly different from the type of inhibition caused by such commonly used immunosuppressants as cyclophosphamide [22, 23] or X-irradiation [20]. Because of this unique suppression, we believe further clinical testing of these compounds as potential antitumor agents is warranted.

sequently funded) had appeared in a review article entitled "Tumour Dormancy" by A. E. K. Alsabti (*Journal of Cancer Research and Clinical Oncology*, **95**, 209; 1979) which had also appeared in the Czech journal *Neoplasma* (26, 351; 1979).

Professor Wheelock said earlier this week that he was hoping to persuade each of the journals to publish a correction. He said that Dr Alsabti had worked in his laboratory for a period of five months but that he had asked him to leave after a disagreement about the authenticity of some experimental data.

Another case in which Dr Alsabti's authorship is questioned is his article "Diagnosis of serum lipids in hepatoma", published in *Oncology* (36, 11 1979). This so resembles an article by Yoshida *et al.* in the *Japanese Journal of Clinical Oncology* (7, 15; 1977) that the editor of the journal has written to *Oncology* saying "I was shocked by the appearance of Dr Alsabti's article which seems a copy of that by Yoshida *et al.* . . .". A copy of this letter has been seen by Dr J. Moglivi of the Anderson Medical Center in Houston, Texas, who was for seven months the immediate supervisor of Dr Alsabti during his spell as a volunteer (unpaid) technician there at the end of 1978.

Dr A. Clarke, president of the Medical Center, said on the telephone earlier this week that Alsabti had come to work in Texas on the recommendation of a Jordanian friend of the hospital but that in the end he was dismissed as a volunteer because of reports reaching the hospital of his exaggerated claims about the work that he had been doing.

One of the referees to whom the paper by Wierda *et al.* was sent by the *European Journal of Cancer* was Dr J. A. Gottlieb of the Anderson Center at Houston. Dr Alsabti was at the center towards the end of 1978. Dr Gottlieb had died some time before.

Index Medicus records that Dr Alsabti published 13 articles in the scientific literature during 1979 and ten in the first five months of this year.

Drug regulations Signs change

Washington

The drug industry has won a measure of support from the General Accounting Office in its complaint that the bureaucracy takes too long to license new products. In a report published last week (6 May), and based largely on comparisons with licensing practice in other industrialised countries, the GAO says that American practice is "lengthy" and that this circumstance "delays the benefits important drugs can provide to the public".

The fact that a new drug application takes on the average 20 months between the submission of test data and the receipt of licensing approval has been a hot potato in Washington for almost ten years. Without making any explicit judgement on the time needed to ensure that the scientific data is adequately reviewed, the GAO report does echo what many pharmaceutical companies have been saying for the past decade.

Excessive regulation, they claim, has not only escalated the costs of bringing a new drug to the market — now estimated at an average of \$62 million — but has led to a growing proportion of their research being conducted outside the United States in countries with easier licensing regulations.

The Food and Drug Administration accepts that its licensing process is lengthy and has taken steps to accelerate the scientific review process. Two years ago, for example, it committed itself to reducing the time taken to license important new drugs by 25 per cent a year over three

successive years, and claims to be on target. But the charges continue that the FDA is not doing enough. And last week congressmen keen further to speed the process quoted the GAO's conclusion that, based on a comparison of the time taken to license fourteen important drugs in six countries, the United States was slower than most in all but one case. According to the GAO, whereas it took on average five months to have a new drug approved in Great Britain and sixteen in Canada, the average time in the United States was 23 months, exceeded only by Sweden's 28 months. FDA counters with its own statistics. Analysis shows, it says, that "the few important drugs that genuinely advance medical care . . . tend to be approved today at reasonably similar times (generally within a few months) in most developed countries".

In response to the charge that its review procedures are too stringent, the agency replies that "of all new molecular entities [drugs whose active ingredient has not previously been marketed in the US] introduced into world medicine in the past decade, no country has approved more than 50 per cent of the total".

Behind the numbers game lie deeper arguments that illustrate how the time taken to approve new drugs is determined as much by the way that the United States has chosen to regulate the drug industry — with a heavy emphasis on administrative record and documented evaluation — as on the adequacy of particular regulations. Pointing to European countries, for example, where independent advisory committees can provide a buffer between a regulatory agency and the industry, the GAO suggests similar expert committees might be used more to review and approve new drugs in the United States.

The FDA disagrees. It says that the open nature of regulatory decision-making in the United States, the right of individuals to sue the government over regulatory actions and the powerful role of congressional oversight each make it difficult to go beyond the thirteen advisory committees now in place.

Another issue is that of post-marketing surveillance. The GAO report points out that in countries such as Great Britain with a national health care system, close contact between doctors and the health services encourages feedback and limits the potential dangers of premature licensing. The FDA, however, has very limited authority to take action on a drug once it has been released, and thus tends to be more cautious before giving licensing approval. There are also suggestions that physicians and hospitals may be dissuaded by the fear of increased medical liability from reporting their experiences.

Tighter provisions for post-marketing surveillance, including in particular the requirement that manufacturers should oblige doctors to notify them of any adverse side-effects, are a central feature of

Two figures — Wierda *et al.* bottom left

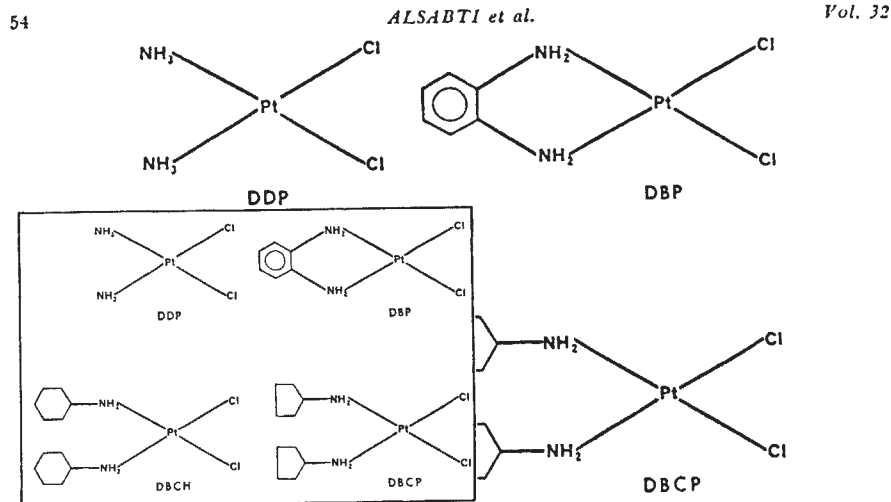


Fig. 1. Chemical structures of *cis*-dichlorodiammine platinum (DDP), dichloro 1,2 benzene-diamine N,N' platinum (DBP), *cis*-dichlorobis(cyclohexylamine) platinum (DBCH), and *cis*-dichlorobis(cyclopentylamine) platinum (DBCP).

two drug regulatory reform bills currently under joint consideration in the House of Representatives.

Both bills are intended to speed up the licensing process, one introduced by the Administration, the other drawn up by Senator Edward Kennedy and passed last autumn by the Senate. For example, an arbitration process would be set up for scientific disagreements between the FDA and potential licensees.

Both bills meet the concern of the FDA that, in particular, it should be given greater flexibility in licensing. They propose, for example, that the review procedure should be speeded up for exceptionally important new drugs and that licences should be issued restricting the use of a drug to specified circumstances where general release might be undesirable.

The pharmaceutical companies are, in general, keen on such revisions. Consumer groups are less happy. "Greater licensing flexibility for so-called breakthrough drugs could provide the camel's head under the tent as far as weakening the general protection provided by the drug laws", says Ben Gordon of the Nader-affiliated Health Resources Group.

But the most important factor may prove to be a recent arrival, namely growing congressional enthusiasm for moves to stimulate industrial innovation in the face of declining productivity and increasing foreign competition, Japanese in particular.

The wish to meet this threat may prove the most potent weapon in the regulation reformers' armoury.

David Dickson

Comecon

Links with West

East-West scientific exchange, except at the Academy/Royal Society level, tends to become associated with other, not strictly academic, matters, as no fewer than three events in London during the past fortnight have demonstrated.

The first, a working visit of the Polish Minister of Metallurgy, Franciszek Kaim, resulted in the signing of two contracts between his ministry, on the one hand, and the British Steel Corporation and the British Metallurgical Producers Association respectively on the other. Although these agreements deal mainly with highly specific themes, such as new technologies for producing coke from non-coking coal, and the perennial theme of energy and raw materials saving, Mr Kaim indicated that on the Polish side at least, a great number of institutes and "other organizations which specialize in scientific research" would be involved in working out "ways and means".

At a later stage, Kaim said, there might be joint research between UK and Polish scientists, both in developing new types of steel and in basic metallurgical research.

Environment, too, he stressed, was a subject where there could be "very effective joint cooperation". (This is a sensitive issue for his ministry — only a few days before his visit, workers at the giant Lenin Steel Mills at Nowa Hut had passed a resolution that existing environmental protection measures there were insufficient.)

This was the third such UK-Polish agreement to be signed — one on mining was signed some three years ago, and one on electricity in February 1980. It comes within the framework of the Joint Commission on Trade and Technology (a linkage that arises, at least in part, from the way in which the former UK Ministry of Technology was dismantled). So did the GDR Engineering Week in Britain (2-5 June 1980).

The East Germans, however, are far less happy about the linking of technological exchange with trade, and are constantly pressing for separate international agreements to cover both. Yet for the purpose of the "Week", Dr Gerhard Beil, Secretary of State in the Ministry of Foreign Trade of the GDR, was prepared to waive these demands — the objective, he said, was "to deepen economic, scientific and technological relations".

The mighty instrument firm of VEB Zeiss-Jena makes no distinction of this kind. After a comprehensive lecture on its latest measuring instruments for the metallurgical industry, the Zeiss delegates made it quite clear that there is no distinction between the scientific and industrial instruments produced by Zeiss in the minds of the 4,000 scientists on the staff.

The third event in the past two weeks had, by contrast, little or no commercial application. Bulgaria is at present preparing for next year's celebrations of 1300 years of statehood, and, in the past few years, has been doing some quite remarkable work in molecular biology. As part of the celebrations, therefore, last week, at the Bulgarian Embassy in London, Sir John Kendrew, in recognition of his services to molecular biology, was invested with the Order of the Madara Horseman and, at the same time, made an honorary foreign member of the Bulgarian Academy of Sciences.

Vera Rich

Biotechnology

Canada stirs

Anxious not to be left behind, the Canadian government is setting up an independent working group to tell whether and how it should promote the growth of biotechnology.

The nine members of the working group are to report back within a year to the Minister of State for Science and Technology, Mr John Roberts, who announced the government's decision on Tuesday at a meeting of the Chemical Institute of

Canada.

The chairman of the group is Dr Maurice Brossard, director of research at the Institut Armand Frappier in Laval, Quebec. Of the remaining eight members, three are from universities and five from industry; others, including government scientists, will be involved as consultants. Although one objective of the working group is to assess the potential of Canada to compete directly with other countries in fields such as the biological production of industrial chemicals and health care products, it is hoped that the group will pay special attention to Canada's characteristic resources and needs — in fields such as energy, mining, food, agriculture and forestry.

Accompanying Mr Roberts's announcement was the publication of a background paper prepared by Dr Louis Slotin, secretary of the committee, listing current Canadian research efforts in biotechnology, broadly defined to include genetic and cellular manipulation, enzyme technology and fermentation techniques.

The report reveals a moderate amount of effort, with total support amounting to about several million dollars a year. But it tends to be thinly spread. The 100 university research workers listed, for example, are distributed among 22 universities; and of the 33 companies indicating an interest in biotechnology research, only ten have more than one or two research workers involved.

To a certain extent Canada makes up in quality what it lacks in quantity. Scientists in the National Research Council's Division of Biological Sciences, for example, are acknowledged to be among the world's leaders in the study of the insulin gene; and several prominent Canadian biologists are already employed as advisers and consultants to US-based biotechnology companies.

The most serious difficulty ahead is a familiar one for Canada — how can an industrial sector largely dominated by foreign-based companies — often with their main research facilities in the United States or elsewhere — be encouraged to support research in Canada and to use what is already being done? There is also a gulf between university research and its potential applications. No Canadian university has a department of applied microbiology, while microbiological research is carried out almost exclusively in medical schools.

A broader issue for the working party will be that of government policy on innovation and whether it should provide direct support for broad-based industrial initiatives — as recommended in Britain's Spinks Report — or instead to follow US strategy with tax incentives and the like.

The inclination of both scientists and industrialists is towards less rather than more government involvement but with a different industrial structure and a smaller supply of individuals prepared to make

high-risk investments, some judicious mixture of private and public involvement seems the most likely recommendation.

David Dickson

Radiation limits

EEC rules soon

A new and legally binding directive on radiation protection for those occupationally at risk, based on recommendations which have provoked controversy in the United States, may be adopted by the European Community within two months. But in contrast to the situation in the United States (*Nature*, 5 June, p350), opposition to the proposed directive has been sporadic and individual.

The European Community has been preparing for a uniform treatment of radiation

safety for several years. In 1976, the Community adopted a comprehensive directive on the subject which was to have come into force in 1978. But a publication of the independent International Commission on Radiation Protection (ICRP 26) caused the Community to have second thoughts.

ICRP 26 recommended a thorough revision of the principles on which radiation exposure should be limited; and the more recent ICRP 30 has derived from these principles the limits on intake of radionuclides implied by ICRP 26 in the light of up to date information on the uptake, retention and effects of radionuclides on the body. The new recommendations differ from those earlier in force by allowing for the risk from an ingested nuclide to all the organs of the body, not simply to the "critical" organs primarily at risk.

The proposed EEC directive takes the views of ICRP into account, and according to an EEC official "differs only in minor detail" from the recommendations of ICRP 26. It would become binding on member states 30 months after adoption.

One feature of the calculations of ICRP 30 is that some of the recommended limits on the rate of ingestion of particular radioactive nuclides are changed by large factors of up to 300; some limits would be more stringent, others less stringent. The US Nuclear Regulatory Commission has argued that it would be impolitic to adopt less stringent limits.

However, the chairman of the committee that prepared ICRP 30 (and its principal author), Dr Jack Vennart of the MRC Radiobiology Unit, Harwell, UK, says that it was incumbent on ICRP to take full account of up to date scientific data and of recent understanding of the physiology and metabolism of radionuclides.

In the case of airborne tritium, for example, it was now recognised that its beta emission was of too low an energy to penetrate to the epithelial tissue of the lung (the most susceptible tissue to cancer). This and other factors resulted in the recommended permitted airborne concentration rising to $2 \times 10^{10} \text{ Bq m}^{-3}$. By contrast, the recommended limits on tritiated water are more stringent because of the likelihood that it will quickly distribute itself throughout the body (whereas tritium gas is poorly absorbed). The ICRP 30 limit on gaseous tritiated water is thus $8 \times 10^5 \text{ Bq m}^{-3}$.

Vennart also points out that it is not ICRP's role to set regulations; that is the business of the relevant national and international legislative bodies. ICRP in fact indicates what seems scientifically the case, and then recommends that levels be set "as low as reasonably achievable, economic and social factors being taken into account". So ICRP is strictly not recommending legislated rises in any exposure level. Vennart believes it unfortunate that "politicians simply adopt the figures wholesale".

Individual criticisms of the ICRP documents in the UK have centred mainly on ICRP 26, which sets radiation — rather than radionuclide — dose levels. Professor Joseph Rotblat, emeritus professor at St Bartholomew's Medical School, for example, says that the dose limit of 5 rems per year recommended in ICRP 26 implies that 2-3 per cent of workers would die of radiation-induced cancer if they worked continuously at that limit, on the basis of the ICRP's own figures.

Rotblat also considers that there may be frequent misuse of the concept of "dose equivalent", which sums up weighted contributions from different organs (see below). In practice, exposure will be assumed to be largely in one organ, he believes, although it may be in more; and if that organ has a low weight in the ICRP 26 scheme, unknown and dangerous high

ICRP standards derived

Dose equivalent in ICRP 26 is defined as follows. First, the absorbed dose after exposure to ionising radiation is given by the energy per unit mass deposited by the radiation in the tissue. It is measured in J kg^{-1} , called Grays (Gy). (In the old units, $1 \text{ Gy} = 100 \text{ rad}$.) Because some particles are more potent than others in causing biological effects, the absorbed dose of a given radiation is multiplied by a quality factor — a function of the particle type and energy — to yield the dose equivalent. This is measured in Sieverts (in old units $1 \text{ Sv} = 100 \text{ rem}$).

The dose equivalent limit in ICRP 26 is then defined as a weighted sum of dose equivalents over 5 'organs' of the body (gonads, breast, red bone marrow, lung, thyroid, bone surfaces and 'the remainder'), the weights taking into account the different sensitivities of these organs to given dose equivalents. (For example, for a given dose equivalent to the tissue, the breast is estimated to be five times more likely to become cancerous than the bone surfaces, so it gets a weight five times higher). The sum must be less than 0.95 Sv; and individual tissue doses must not exceed 0.5 Sv.

The limit to the sum (0.05 Sv) is chosen to control 'stochastic effects': in other words the probability of contracting cancer. Assuming all cancers are fatal, and that an average worker in the nuclear industry would receive one-tenth of the dose equivalent limit, the limit is set to produce a probability of death about the same as is already tolerated in other 'safe' industries (about 3 per thousand for a 30-year lifetime). ICRP 26 says that "long-continued exposure of a considerable proportion of the workers at or near the dose-equivalent limits would be only acceptable if a careful cost-benefit analysis had shown that the higher resultant risk would be justified".

The limit to individual tissue exposure

(0.5 Sv, except the eye, whose limit is 0.3 Sv) is designed to eliminate 'non-stochastic effects': cumulative radiation damage which is not lethal and is undetectable below some threshold. (Cataract of the cornea and hair loss are examples.)

ICRP 30 then applies these limits to various radionuclides, taking into account (to a much greater extent than ICRP 2) their chemical form, and transmissibility from gut to bloodstream and from lung to bloodstream (which depends on particle size).

This results in an 'annual limit of intake' (ALI) which is the intake that would cause the dose equivalent limit of radiation to be reached; and the 'derived air concentration' (DAC) which is the concentration which, breathed each week for 40 hours, leads to the absorption of the ALI.

Comparisons of ICRP 30 with ICRP 2 are not direct, because the isotopes are divided into chemical classes in different ways (ICRP 2 distinguishes only 'soluble' and 'insoluble' for example) and ICRP 2 considers limits for each organ separately. However, its DACs derived from ICRP for soluble and insoluble compounds of an isotope can be compared.

Here are a few examples in Bq m^{-3} . ($1 \text{ Becquerel} = 1 \text{ s}^{-1} = 2.7 \times 10^{-11} \text{ Ci}$.) In some cases only one value is quoted.

Radionuclide	DAC, ICRP 2	DAC, ICRP 30
^3H as $^3\text{H}_2\text{O}$	2×10^5	8×10^5
^3H as $^3\text{H}_2$	8×10^7	2×10^{10}
^{60}Co	360-12,000	50-3,000
^{90}Sr	12-200	60-300
^{131}I	360-12,000	700
^{137}Cs	400-2,400	2,000
^{226}Ra	1.2-8,000	10
^{239}Pu	0.08-0.16	0.09-0.3

Some of these figures make ICRP 2 more stringent, some ICRP 30.

Robert Walgate

exposures to it may be allowed while exposures of other organs may be being ignored — the exact opposite of the intention of the weighting scheme. Similar arguments have been raised in the Swedish National Institute for Radiation Protection.

Robert Walgate

Cosmonauts

More and merrier

ON 26 MAY, Bertalan Farkas of Hungary became the fifth non-Soviet citizen to go into orbit aboard a *Soyuz* spacecraft. With more than half the Comecon allies now accounted for, Soviet space planners are stressing that hospitality aboard their space-stations is theoretically open to countries outside the Socialist bloc. France and India are already on the waiting list. The possibility of wider participation in the Soviet space programme was advertised last February at the Hamburg Scientific Forum by Academician Nikolai Blokhin.

For the Comecon guests aboard the *Soyuz* and *Salyut* spacecraft, a certain pattern of experimental work has emerged. As well as taking part in routine biomedical and astrophysical observations, the participating country also contributes a set of experiments reflecting its own particular interests. It further contributes a crystallography experiment, in which a hermetically sealed ampoule containing a mixture of compounds that will not normally crystallize homogeneously under gravity is treated in a furnace aboard the *Salyut* station in the hope of producing new semi-conductor substances. Traditionally the experiment is given a name symbolizing the national traditions of the country concerned. For Hungary, the name chosen was *Otvos* (Goldsmith), referring to an ancient Hungarian craft, and also a tribute to Lorand Eotvos (the pronunciation is the same), the nineteenth century Hungarian physicist.

More specifically Hungarian in origin are the thermoluminescent dosimetry experiments *Integral* and *Pille* (Moth). The Central Research Institute for Physics of the Hungarian Academy of Sciences has been interested in the thermoluminescent monitoring of ionizing radiations for more than 15 years, and since 1970 has contributed dosimetry instruments to the *Interkosmos* programme.

The *Integral* test measures accumulated radiation at various points of the spacecraft over several months. The first test run was inaugurated aboard *Salyut-6* in 1979, and a second set of monitors (crystalline samples of $\text{CaSO}_4:\text{Dy}$ and $\text{BaSO}_4:\text{Tm}$) have now been installed. *Pille*, a miniaturized instrument by Comecon standards (1 litre volume, 1 kg mass, and with a dosage range from 10 mrad to 10 rad), is a short-term monitoring device, giving readings of accumulated dose every 2-3 days. The same type of dosimeters will ultimately be used for monitoring the personnel of the Paks nuclear power



Farkas — looking alike

station, now under construction in Hungary.

Photography and telemetry of the participating country is also now a traditional part of joint flights. For Hungary, this has meant geodesic surveying (to check existing maps and optimize land use), photographing the whole of the Hungarian section of the Danube (preparatory to monitoring environmental changes expected after construction of the hydroelectric plant at Nagymanos and the nuclear power station at Paks) and the observation of eutrophication and pollution of Lake Balaton and its tributaries.

Farkas was given charge of a series of experiments intended to throw light on the production of interferon by leucocyte cultures, but more practical considerations have not been neglected. Apparently sceptical of claims in 1978 by the Soviet-Polish mission that cosmonauts lose their sense of taste, Farkas was provided with special picnic packs by the Quartermasters' Division of the Hungarian People's Army and the Research Institute of the Cannery and Paprika Processing industry. The packs included goose-liver pate, pork-paprikas, bean salad with sausage and chicken in aspic, and were designed so that Farkas could share them with his hosts.

Vera Rich

UN assistance

No rows yet

Washington

As UN events go, last week's meeting in New York of the new Inter-Governmental Committee on Science and Technology for Development was a relatively tame affair. There were no late-night sessions, and little of the international tension that pervaded last year's UN conference on the same theme in Vienna.

But if the outcome of the meeting can be weighed more easily in terms of bureaucratic prose than in targeted research

dollars, many third world delegates returned home confident that they had helped to steer the scientific activities of the UN system closer to their self-perceived needs.

Set up by the UN General Assembly on the recommendation of the Vienna conference, the IGC provides a forum on science policy matters for all member states of the UN — and is thus an implicit move to shift the control of such matters away from groups more heavily dominated by the developed countries (as the now-defunct Office of Science and Technology was perceived to be).

The committee has been created by another new creation, the Centre for Science and Technology for Development. Two days before the New York meeting, the director of the centre — who has the rank of assistant secretary-general — was named as Dr Amílcar Figueira Ferrari, an engineer who was director of Brazil's National Research Council and a member of that country's delegation to UNCSTD.

Some of the developed countries made little secret of their frustration with the meeting's emphasis on administrative matters, urging that work should start on specific projects. The Canadian delegation, for example, put in a strong bid for the setting up of an international scientific and technological information system and were disappointed when the IGC decided that organisational and institutional arrangements should take precedence.

Most delegates from the developing countries, however, expressed the view that the coordination and control of research efforts within the UN system — as well as efforts to stimulate national and regional research programmes — may prove of more long-term significance than attempts to raise additional research funds from the industrialised nations, particularly at a time of general financial stringency.

The New York meeting faced three particular issues: how to set in motion the main recommendations of the Vienna conference; how to define the role of the Centre for Science and Technology with respect to the Interim Fund for Third World Science established and administered by the United Nations Development Programme; and how to approach the thorny question of 'harmonising' the scientific and technological efforts of the specialised UN agencies.

On the first issue, there was little contention. A new Scientific Advisory Committee will be set up, a successor to the Advisory Committee on the Application of Science and Technology to Development (ACAST) serviced by OST. And member countries of a 27-member group of experts were named to report on the long-term prospects for a new science and technology financing system within the UN.

On the second issue, there was a rerun of debates in Vienna, with some Third World delegates demanding greater control by the new centre over the UNDP Fund — for

which \$36 million in contributions and another \$10 million in commitments was raised two months ago — while the developed countries and UNDP officials sought maximum flexibility.

In the end, a compromise was reached under which the centre will be given a role in 'reviewing' the operation of the Interim Fund. In addition new guidelines for the fund were agreed; some delegates, for example, were critical of the fact that a

large proportion of the 350 funding proposals received had originated from within UN agencies, and stipulated that all proposals for funding should come through governments. But the IGC's role remains one of direction-setting and overview rather than direct involvement.

Coordinating the scientific and technological activities of the 'organs and organisations' within the UN system will prove the hardest nut to crack. Most of the

specialised agencies, such as the United Nations Educational Scientific and Cultural Organisation, are fiercely protective of their independence, resisting any effort to subsume this independence under any centralised mechanism.

But third world delegates on the IGC remain adamant that increased co-ordination between the agencies is essential if the broad goals of the Vienna conference are to be met.

David Dickson

CORRESPONDENCE

Split syntax

SIR,—While Dr Glascock is to be congratulated on his ingeniously contrived verse on syntax (8 May, p.66), he should have been more careful in the way he worded his introductory paragraph. As the Hungarians say, "Bagoly mondja verébnek hogy nagyfejű".*

I therefore offer the following supplementary stanza:

You are right Doctor Glascock to chide as you do
The scientist for what he has writ
But one rule of syntax eludes even you:
Infinitives should never be split!
Yours faithfully,

G A GARTON
(With but scant acknowledgements to Fowler)
Aberdeen, UK

*"The owl calls the sparrow a bighead."

No room in the ark

SIR,—Impetuosity in science is surely to be avoided in favour of caution even as exaggeration is to be eschewed in favour of understatement, but do these wise maxims excuse Mr Thomas H. Jukes for his unconscionable conservatism in his short piece "Two By Two" (*Nature* 15 May). Commenting on the Noachian flood, Mr Jukes opines that Noah's ark would have provided less than one cubic metre, on the average, for each pair of vertebrates plus their food, a sufficient supply of which was needed to last for about a year. But why, we may wonder, did he undertake his calculations on the basis of *Genesis* 6; 19 in which Noah is directed to take aboard the ark a male and female pair of every living thing rather than *Genesis* 7; 2-3 wherein Noah is directed to take aboard seven pairs of all clean beasts and fowls of the air in addition to one pair of unclean beasts, fowls and creeping things plus food for all?

Mr Jukes would do well to read the eleventh chapter of *Leviticus* (in which clean beasts, fowls and creeping things are distinguished from unclean ones) before recommencing his calculations. Whatever the results of that unenviable task may be, one cubic metre per pair of vertebrates will be excessively capacious.

Even less excusable is his calculation that 393 million cubic miles of water resulted from the flood, a figure at which he arrived on the entirely arbitrary depth of 10,000 feet. Nor was there the slightest reason for him to compare that admittedly conservative figure with 17,000 feet, the approximate elevation of Mt Ararat. *Genesis* 6; 19-20 asserts that the waters rose fifteen cubits (about twenty-two feet) above all the high hills and the mountains of the earth. That would mean that even Everest was submerged and would involve a

depth of nearer 30,000 than 10,000 feet, making 393 million cubic miles of water a mere puddle by comparison.

Is it possible that Mr Jukes, despite his air of wry scepticism, is really an apologist for scientific creationism or, perhaps, even a member of the Creation Research Society? If so, he will know how to handle the problems he raises such as the waste "disposal problem" on the ark, the "botanical problem" after the deluge, the "bacteria and protozoa" collection problem and the water dispersal problem. It is on this very point that modern profane science founders, utterly incapable, it would seem, of relying on the miraculous to solve thorny problems, theoretical or otherwise. Creation science, on the contrary, knows no such incapacity but invokes the miraculous at will, often and gladly.

Yours faithfully,
DELOS B. MCKOWN
Department of Philosophy
Auburn University, Ala., USA

SIR,—In Mr Jukes's article entitled "Two by Two" (*Nature*, 15 May) his most plausible argument against the biblical account of the Flood appears to lie in his last paragraph. His listing of amphibians, bacteria and protozoa as having to be gathered into the ark is open to question, and he has not mentioned the possibility that all the animals there might have been young and not mature specimens.

But his calculation of the rainfall needed to produce a universal flood, and the means of ultimate disposal of the water being held up to doubt, appear impressive only until one sees that he has overlooked the first cause of the flood. *Genesis* 7; 11 states: "The same day were all the fountains of the great deep broken up, and the windows of heaven were opened." Rain supplied only part of the flood water.

Second, Mr Jukes appears to assume that the relative heights and depths of the earth's surface before the flood were as they are now, an assumption which is in no way logical. That

great changes in the elevations and depressions of the land and seas have occurred is clear from, for instance, the burial of coal measures below thousands of feet of sedimentary rocks and the existence of submarine canyons. Radical change after the flood is suggested by another scriptural reference *Peter*, 2; 5 and 6.

It has been suggested that the statement made in *Genesis* 1; 6 about waters above the firmament indicates that there was above the earth a canopy of water vapour which was precipitated during the flood. This would account for a change in salinity of the oceans which in a recent article in *Nature* was mentioned as a possible cause of the dying out of the dinosaurs

Yours faithfully,
D. CONWAY
Weymouth,
Dorset, UK

Cheaper in paper

SIR,—From the Spring Book issue of *Nature* (24 April) I have taken at random the prices of a dozen books issued in both forms. The ratio (paperback price)/(hardback price) ranges from 0.352 to 0.607 and the average for the dozen books is 0.439. From the list of books, it can be seen that the books are not confined to books on any one subject or to books only of broad popular interest.

The books I have considered are scientific and technological. Whether there are similar difference for books in the areas of the arts and humanities, I do not know. I hope that publishers of scientific books will make paperbacks more widely available, thereby increasing sales and perhaps even profits as well.

Yours faithfully,
G. W. BRINDLEY
Pennsylvania State University
University Park,
Penn., USA

	Prices		Ratio
	Hard cover	Paperback	
Science and technology (Freeman)	£ 8.90	£ 5.40	0.607
Early diagenesis (Princeton)	£13.70	£ 5.25	0.383
Evolution of the igneous rocks (Princeton)	£19.30	£ 8.40	0.435
Evolution of North America (Princeton)	£15.20	£ 5.35	0.352
Knowledge and wonder (M.I.T.)	\$15.00	\$ 5.90	0.393
Mesozoic mammals (U. California)	\$21.00	\$ 5.75	0.274
Peano (Reidel)	\$34.00	\$14.95	0.440
Nuclear power and public policy (Reidel)	\$19.95	\$10.50	0.526
Serengeti lion (1972) (U. Chicago)	£12.25	£ 5.60	0.453
Condensed matter physics (Addison-Wesley)	\$26.50	\$14.50	0.547
Practical methods in electron microscopy (Elsevier)	\$73.25	\$27.50	0.375
Coulson's valence (Oxford)	£17.50	£ 8.50	0.485
	Average ratio		0.439

Chromosomal action of ecdysone

from Michael Ashburner

THE hormonal control of growth and metamorphosis in insects was discovered over 60 years ago by the Polish biologist Stefan Kopec¹ but it was not until 1965 that the chemical identity of the hormone, by then called ecdysone, was established². A few years before the final chemical characterisation of ecdysone, Clever³, in collaboration with Karlson, showed that ecdysone was able, in a period of only a few minutes, to induce puffs in the salivary gland polytene chromosome of the midge *Chironomus*. They put forward the then controversial interpretation that this represented a change in the pattern of genetic activity. It is only recently that direct evidence has shown that the puffs of polytene chromosomes are, indeed, active genes. In *Drosophila*, this has been demonstrated from the changing patterns of protein synthesis that follow changing patterns of puffs⁴ and from the subsequent molecular characterisation of the genes involved⁵. Several of the genes concerned with the synthesis of the salivary gland's secretion have been mapped and shown to be coincident with puffs active at the time of the synthesis of these proteins⁶.

Clever and Karlson's discovery was the first clear indication that hormones may act directly on the genes themselves, an idea that has been very fruitful for the study of the mode of action of many vertebrate steroid hormones. The induction of specific genes by ecdysone inevitably lead to interpretations of the mechanism of the hormone's action based upon the then novel ideas of the control of gene activity in *E. coli*. The reaction against this rather simplistic view was not long in coming. Kroeger⁷ suggested, on the basis of the results of experiments with explanted *Chironomus* salivary glands, that what ecdysone did was to control the internal ionic milieu of the cells and their nuclei and that the genes responded to the hormone via an altered ionic environment. In particular, Kroeger suggested that ecdysone acted on the cell's membrane to effect an increase in the intracellular potassium ion concentration and that the ecdysone responsive genes were sensitive to this change.

For almost seventeen years the controversy between Kroeger and his colleagues and those who believed in a rather more direct action of ecdysone on the genes continued unabated. In the last year or so, two rather different types of experiment have yielded data that are capable of both resolving this particular conflict and, more importantly, of giving some insight as to the molecular basis of the modulation of genetic activity by ecdysteroid hormones.

Although ecdysteroids were the first hormones to be shown to have a very rapid effect on genetic activity, the study of the vertebrate steroid hormones, particularly oestrogen and progesterone, rapidly outpaced that of the ecdysteroids. This arose from the discovery that responsive cells possess specific 'receptor' proteins, that bind the hormone with high affinity and then move to the nucleus. There is good circumstantial evidence that receptor protein binding and translocation to the nucleus are obligate steps in the hormone's action and that the receptor complex binds to specific chromosomal sites, causing specific changes in gene expression.

Evidence for a similar class of receptor protein in ecdysteroid responsive tissues was difficult to find. The reasons for this were largely technical. Radiolabelled ecdysteroids of both a high enough specific activity and high enough biological activity were simply not available. It was only when Fristrom and O'Connor realised that a highly radioactive ligand, known as ponasterone A, could be prepared from a plant ecdysteroid, stachysterone C, that progress was made possible. Ponasterone A shows even greater biological activity than 20-OH ecdysone itself. Using this radiolabelled ecdysteroid Yund and Fristrom⁸ and Maroy *et al.*⁹ discovered ecdysteroid receptor proteins in *Drosophila* imaginal disc cells and in a permanent *Drosophila* cell line respectively.

These ecdysteroid receptors are similar to those for oestrogen or progesterone in

vertebrates; they are proteins which bind the hormone with high affinity and high specificity, the binding constants for different ecdysteroids differing just as these hormones differ in their biological activities. The hormone-receptor complex enters the cell's nucleus though, in contrast to vertebrates, this does not involve a temperature dependant activation step.

Although the ecdysteroid receptors of *Drosophila* have yet to be purified, evidence for their activity comes from the study of *Drosophila* cell lines. Many cell lines of *Drosophila* respond to low concentrations of ecdysteroid in a complex and pleiotropic manner. In derivatives of the Kc cell line, for example, this includes the induction of specific enzymes, such as acetyl cholinesterase¹⁰ and what appears to be an inhibition of cell division. This latter effect means that any variant cells capable of resisting the hormone's effects will be selected very rapidly from a population of largely responsive cells^{12,13}. Preliminary reports¹⁸ indicate that certain ecdysteroid resistant clones of *Drosophila* cell lines lack hormone binding activity.

The eventual purification of the ecdysteroid receptor proteins will open up many avenues for research and allow direct study of the location of ecdysteroid action on the genome. However this information can be obtained in another way.

The ecdysteroids contain an α - β unsaturated ketone in the B ring of the nucleus. Gronemeyer and Pongs¹¹ realised that this makes it possible to photo-cross-link ecdysteroids to their cellular binding sites. Using the salivary gland polytene chromosomes of *Drosophila* they irradiated the cells with light of > 320 nm to carry out the cross-linking; the hormone was visualised by first treating the chromosome with an anti-ecdysteroid antibody and then 'staining' with a fluorescent tagged goat anti-rabbit antibody. Despite a low background fluorescence, the polytene chromosomes show specific regions which fluoresce very brightly. When these regions are analysed in detail they are found to correspond to those regions that respond to ecdysteroids by forming puffs.

The nature of the substrate to which the

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photo-cross-linked ecdysteroids are bound is unknown. It may be the ecdysteroid receptor protein itself or some other chromosomal protein. The fact that this substrate can be covalently bound to the hormone, and that the hormone can be labelled to a high specific activity suggests that this problem will not be too difficult to solve. Moreover a comparative study of, for example, hormone responsive and hormone resistant cell lines of *Drosophila* should afford a direct demonstration of the role of nuclear receptor proteins in the hormone's action. It should also be possible to isolate mutants of *Drosophila*, rather than just their cell lines, that are mutant for their receptor proteins¹⁵.

The cloning of the ecdysteroid responsive genes of *Drosophila* is a particularly exciting prospect. Evidence from both polytene chromosomes^{3,14} and tissue culture cells¹³ suggests that complex

pleiotropic responses to the hormonal stimulus are mediated via a few key genes. In salivary gland polytene chromosomes, for example, only six or so genes are induced by ecdysteroids within the first half hour or so. The induction of these puffs is independent of protein synthesis. A very much larger number of puffs respond after a few hours lag and their induction requires protein synthesis, at least during the time the early puffs are active^{16,17}. Analogous events occur in *Drosophila* cell lines. A very early effect of ecdysteroids is to induce the synthesis of two or three polypeptides. Cherbas¹³ and his colleagues have recently isolated cDNA clones complementary to the mRNAs of these polypeptides and found that the induction of these mRNAs, like that of the early puffs, is independent of protein synthesis.

A most striking characteristic of ecdysteroid's action is its tissue specificity. The different tissues of a *Drosophila* larva respond to the hormone in many different ways. The cloning of the 'early' ecdysteroid responsive genes from different tissues, for example fat body, imaginal discs and salivary glands, will allow us to discover whether or not these different tissues use the same genes as the primary targets of the hormone^{19,20}. These clones will, now the outlook for the purification of the ecdysteroid receptor protein is so bright, result in a very detailed understanding of just how this simple hormone acts to effect the changes in gene activity that eventually result in the metamorphosis of the insect. □

Such strains were first produced by D. Bailey (Jackson Laboratories, Bar Harbor, US). RI mouse strains are, in effect, permanent segregant populations that frequently eliminate the need for slow and tedious backcross linkage analyses for the mapping of genes. Two resistance genes have been mapped using RI mice. The first is a gene locus, *Lsh*, that controls the intrahepatic and intrasplenic growth of *Leishmania donovani*, the etiological agent of human visceral leishmaniasis (D. Bradley, London School of Hygiene and Tropical Medicine, London). The *Lsh* locus has been mapped to the proximal end of chromosome 1. The second locus that was mapped using RI strains, is *Ric*, a gene that controls resistance to lethal infection with *Rickettsia tsutsugamushi*, the etiological agent of human scrub typhus. The *Ric* locus has been mapped to the middle of chromosome 5, closely linked to the gene for retinal degeneration, *rd* (M. Groves, Walter Reed Army Institute of Research, Washington DC). *Ric* seems to control a very early response in infected mice, since its effects can be very clearly demonstrated within four hours of inoculation of infectious organisms.

A third resistance gene that has been mapped controls resistance to lethal infection with *S. typhimurium*, and the locus has been termed *Ity* (for immunity to *typhimurium*). It is interesting to note that *Ity* is probably identical to the gene that was first inbred by Webster. *Ity* was mapped using a more traditional linkage analysis, and is located fairly close to *Lsh* on chromosome 1 (J. Plant, St Mary's Hospital Medical School, London). Although the close linkage between *Lsh* and *Ity* suggests that these loci might be identical, several RI strains have been identified in which alleles of these loci are not concordant, indicating that the genes are closely linked but different (A. O'Brien, USUHS, Bethesda).

A second major theme of the meeting was the mechanism of action of various resistance genes. Although no one has as yet pinned down a specific biochemical defect, some progress in this area is evident. An unexpected finding was the role of organism specific suppression in producing susceptibility. The disease produced by *Leishmania tropica* (human cutaneous leishmaniasis) is a good example of the operation of such a suppressive mechanism (J. Howard, Wellcome Research Laboratories, Beckenham, UK). Susceptibility in this mouse strain is due to a single, autosomal co-dominant locus. In response to *L. tropica* infection, Balb/c mice develop a population of antigen — specific T suppressor cells that prevent the development of an effective cell-mediated immune response of the type that

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Genetics of resistance to infection

from David L. Rosenstreich

HUMAN populations vary markedly in their susceptibility to disease produced by infectious agents. There are abundant examples of this variability, ranging from the well documented to the anecdotal. An example of the former is the resistance of erythrocytes containing Hgb S to infection by *P. falciparum* malaria parasites. Examples of the latter would be the familiar clinical observations of the marked variation between children in the severity of disease produced by highly contagious organisms such as chicken pox. There has always been a strong feeling among medical personnel that some of this variability was genetic in origin. However, with the exception of isolated examples of traits that produce marked biochemical or immunological derangements, the only other well documented example of genetic variability in susceptibility to infectious disease in humans has been the work in the major histocompatibility gene locus, HLA.

However, research with inbred mouse

populations has indicated that a large number of genes control resistance to infectious agents. Studies in this area date back to the 1930s when Webster (*J. exp. Med.* 57, 793; 1933) developed inbred strains of mice that differed markedly in susceptibility to the etiological agent of murine typhoid, *Salmonella typhimurium*, or to arbovirus infection. Over the past four decades, reports describing variations in resistance to other microorganisms in mice have surfaced, so that patterns of variability of resistance to most classes of viral, bacterial and parasitic human pathogens are known to exist in mice. A recent workshop* showed that research in this field has greatly accelerated over the past few years.

One of the major advances discussed at the meeting was the identification of the chromosomal location of specific resistance genes. Gene mapping in mice has been greatly facilitated by the development of recombinant inbred (RI) mouse strains.

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eliminates the organism from resistant mouse strains. Antigen specific suppression in susceptible mouse strains was also shown to occur in response to other organisms including *Mycobacterium bovis* BCG, (R. Nakamura, NIH, Tokyo) and several viruses (E. Isael, Jewish General Hospital, Montreal) and was also found to be involved in the failure of SJL mice to develop natural killer (NK) cells (G. Cudkowicz, State University of New York).

A third important aspect of genetic control of resistance is the effect of resistance genes on the response of organisms to various therapeutic agents. Mice that are genetically susceptible to *S. typhimurium* cannot be protected from this organism by immunization with standard vaccines, in contrast to genetically resistant strains (Rohson & Vas, *J. inf. Dis.* **126**, 378; 1972). Evidence presented at the workshop by H. Perez (IVIC, Caracas) suggests that this problem may extend to responsiveness to chemotherapeutic agents as well. Mice that are genetically susceptible (Balb/c) to *L. mexicana* (mucocutaneous leishmaniasis) did not respond to treatment with the drug, glucantime, while genetically resistant mice (C57 B1/6) could be cured by similar doses.

A workshop on 'Genetic control of natural resistance to infections and malignancy' was held in Montreal 18-20 March 1980 by the Canadian Society for Immunology. The organisers were E. Skamene, P. Kongshavn, P. Gold and J. Shuster.

Findings such as these may be very important in explaining the wide variations in the response of human subpopulations or individuals to vaccines or drugs that tend to be less than 100% effective.

Several other themes were brought out at the workshop. One is that that control of early resistance to many different classes of organisms appears to be expressed in macrophages. Another is that most of these early resistance genes are not linked to the mouse major histocompatibility locus, H-2, one of the major regulators of antigenic specificity. Finally, it is noteworthy that very few of the described resistance genes seem to be identical, and there is some evidence of antigen or organism specificity in the actions of these genes. While subsequent mapping studies may begin to uncover overlapping specificities for some of these genes, these findings strongly suggest that there may exist a whole class of non H-2 linked genes that also control recognition of foreign substances.

The workshop illustrated the interest and progress that is beginning in this recently rediscovered area. The application of genetic approaches to studies of complex functions such as resistance to infections offers the possibility of real breakthroughs in the future. Implications for human health and disease are profound. □

quite faint. However, the observation by Gunn *et al.* (*IAU Circ.* No. 3431; 1979), which showed that there is a slightly elongated galaxy at redshift $z \sim 0.4$ about 0.8 arc s north of the southern (B) quasar, and no object midway between the two images, has changed the picture considerably. If the galaxy near B is indeed the gravitational lens, calculations show that, as a point mass, it is not far enough from the light path to cause the correct light deflection, and so a more complex picture based on an extended (and perhaps non-spherical) mass distribution is required. Under these circumstances it appears that either one or three images are formed, depending on the angles of deflection required, rather than the two images produced on the simple picture. So it is now likely that B consists of two close (≤ 0.2 arc s) unresolved images, and the absence of extended radio structure around B corresponding to that found around A is no longer a problem. The apparent radio extension of B may be due to weak emission from the galaxy itself, and indeed the positional agreement between the two is very good. Asymmetries in the galaxy, or the presence of a cluster of galaxies, could easily account for the slight misalignment of the two images and the deflector.

This new picture resolves some other problems which the simple model fails to explain adequately. Another prediction of the gravitational lens model is that the flux ratio between two images of a compact object should be independent of wavelength. This has been verified at radio frequencies by Pooley *et al.* among others, in the short wavelength optical region in the original paper by Walsh *et al.*, and now in the satellite ultraviolet region, by Gondhalekar and Wilson (this issue of *Nature*). However in the red and infrared part of the spectrum, B appeared to be somewhat brighter than expected compared with A. Recent infrared measurements by Soifer *et al.* (*Nature* **285**, 91; 1980) and Lebofsky *et al.* (*Nature* **285**, 385; 1980) are, however, consistent with a constant flux ratio between the two quasar images if a giant elliptical galaxy with a redshift $z \sim 0.4$ contributes to the excess flux in B.

Thus the suggestion that 0957 + 561 A and B are two (or, now, three) images formed by a gravitational lens has been well tested, and while some of the details of the process are not as simple as first envisaged, the essential correctness of the model is no longer in serious doubt. There are a number of detailed questions which remain, many of which require further observational material that may be difficult to obtain. Comparison of the VLBI radio structure of the two compact sources should yield valuable information. Another direct check on the model is to obtain the velocity dispersion for the material in the lens galaxy to see if it is consistent with the mass and radius required to give the observed deflection of

A year of the double quasar

from a Correspondent

IN this issue of *Nature*, Gondhalekar and Wilson show that satellite observations in the ultraviolet region verify the gravitational lens model of the twin quasars.

It is a little over a year since the discovery of the twin quasars, 0957 + 561 A and B, was reported by Walsh *et al.* (*Nature* **279**, 381; 1979). They found that the two quasars, which are only about 6 arc s apart on the sky, have nearly identical spectra, and they thus suggested that the two images are of a single quasar in a gravitational lens. Since the two images have comparable brightness, it appeared likely that the object deflecting the light would be nearly midway between them.

Since this discovery, a large number of observations have been made on the double quasar at many wavelengths, testing some of the predictions of the gravitational lens model. The remarkable similarity of the optical spectra, which was the first pointer to the model, has been further demonstrated by Weymann *et al.* (*Astrophys. J. Lett.* **233**, L43; 1979) and by Wills and Wills (*Astrophys. J.* **238**, in the press). A further stringent test has come from the radio maps obtained by Pooley *et*

al. (*Nature* **280**, 461; 1979) from the Cambridge 5 km telescope in the UK, and by Greenfield *et al.* (*Science* **208**, 495; 1980) using the Very Large Array of the National Radio Astronomy Observatory in the US. The radio source was found to have a complex radio structure which extended over about 12 arc s. The brighter northern component (A) of the pair is near the centre of an extended nearly linear structure which is at a large angle to the line joining the two quasar images. This has no counterpart in the southern (B) component, which is a compact source with a weak extension pointing approximately towards the A component. The absence of any radio structure near B corresponding to that found near A is difficult to reconcile with the simple gravitational lens model as first proposed.

The other obvious approach to testing the gravitational lens model for the twin quasars is to search for the object, presumably a galaxy, which is causing the gravitational deflection of the radiation. Early attempts to do this were hampered by relatively poor seeing but showed that if a galaxy were present nearly midway between the two quasars then it would be

the radiation in the two images. In principle this could be done for the starlight from the galaxy, though this is rather faint and close to one of the quasar images. Alternatively, we might expect to find redshifted MgII absorption from any gas in the galaxy to determine this quantity, but already, from the spectra given by Walsh *et al.* in the original paper, we know that such lines are fairly weak. No doubt these observations and other detailed work will be done, but it would be somewhat surprising if the overall picture were to be very different from that we have now. □

Transient lunar phenomena

from David W. Hughes

THREE thousand million years is a long time for the Moon to be quiet and cold, but is it completely dead? Probably not, is the most reasonable answer. For more than two centuries terrestrial observers have recorded events which have been grouped under the title of transient lunar phenomena (TLP). The possible causes of these phenomena have always been subject to considerable debate and the latest debator is Allan Mills of the Department of Astronomy, University of Leicester. His review paper is published in a recent edition of the *Journal of the British Astronomical Association* (90, 219; 1980).

Over 1,400 TLP's have been observed and even though a considerable number of these can be rejected as instrumental, atmospheric or physiological artefacts a sufficient residue remain to warrant a detailed physical investigation. Glows, hazes, mists, brief colour changes and temporary obscurations of lunar surface features have been reported. Events seem to be restricted to specific lunar regions, about 300 having been reported from the crater Aristarchus, 75 from Plato and 25 from Alphonsus. They also occur near the boundaries of certain regular maria and near areas rich in rills. The highlands seem to be avoided, but this apparent paucity may be an observational selection effect. Occurrence frequency is not linked with solar activity but does peak when the Moon is at the perigee of its orbit, at times when tidal activity is maximised. Phenomena have been reported from both the sunlit and dark sides of the Moon. The areal extent on the lunar surface is on average 16 km across, this containing brighter spots of between 3 and 5 km in diameter. The average duration is about 20 minutes but some have been reported as persisting intermittently for a few hours. No permanent changes have been observed on the lunar surface after a TLP, thus justifying the use of the word 'transient'. Under very favourable seeing conditions TLP's have been seen to twinkle. Colour, if

mentioned at all, tends to be described as weak, unsaturated, 'reddish' or 'bluish'.

Many possible explanations have been put forward, one of the first by Sir William Herschel who reported observing bright red glows in 1783 and 1787 and wrote a paper entitled "*An account of three Volcanoes in the Moon*". The phenomena looked like glowing charcoal thinly veiled with hot ashes and Herschel was obviously thinking of incandescence from hot lava on the lunar surface. Unfortunately, fresh lava regions have not been seen on the Moon, at Herschel's site or in other places. The visible radiation from the fresh lava would decrease rapidly although the infrared radiation would be much more persistent. No such infrared sources have been observed. The brightness is also too low. 1 km² of lava at 1,250 K appears as an orange-red 'star' of magnitude 5.5. Drop the temperature to 1,000 K and the magnitude becomes 10.5. This might just be visible on the dark side of the Moon but would be very hard to detect on the sunlit side.

Luminescence can be ruled out because the excitation source, the solar wind, is too weak.

Thermoluminescence can also be disregarded because even though it provides a means of storing energy the emission intensity of all known materials is still too weak to be seen from such a distance.

Triboelectric charging can occur when dust grains are rubbed together. Discharging in dust clouds above the Italian volcano Vesuvius during its 1944 eruption produced brilliant and frequent lightning strokes. The emission of light depends to an extent on the presence of gas so that a plasma can be generated. Diffuse glows occur at about 100 dyn cm⁻² these being replaced by bright twinkling discharges as the pressure increases. The potential required to produce a discharge is related to the product of the gas pressure and the dust separation. This process will be discussed again later.

Other explanations for TLP's rely on short term modifications to the lunar surface reflectivity. One reason for the very low reflectivity of the lunar soil is the spiky 'fairy castle' structures formed by the dust grains. If these are flattened out the reflectivity increases and this flattening can be easily produced by fluidizing the upper dust layer by passing gas through it. There are two difficulties. Large quantities of gas are required to fluidize several sq. km. of lunar soil. A more pressing problem is that the enhancement in reflectivity is semi-permanent and this is ruled out by the 20 minute average duration of TLP's.

Middlehurst (*Phil. Trans. R. Soc. Lond. A* 285, 485; 1977) compares the epicentres of deep moon quakes and high frequency teleseismic (HFT) shallow moon quakes with the areas of TLP activity. Most of the HFT sites are within 5° of at least one TLP site. The deep moon quakes originate 800

to 1,000 km below the surface, the shallow ones being much higher (the maximum depth found so far is 265 km). Middlehurst suggests that channels exist between HFT epicentres and the TLP's (the TLP's being almost vertically above the HFT's) and that gas is still escaping through these channels even though the escape rate is much smaller now than it was in the earlier days of lunar history.

Mills proposes a mechanism for TLP production which relies on gas escape but at a much lower rate than that required to fluidize the soil. The gas lifts off the very small particle fraction of lunar soil, producing a kind of moon smoke, and leaves the fairy castle structure intact. Unfortunately the scattering of light by smoke is a highly complex and incompletely modelled phenomena, which relies drastically on the particle size distribution. For particles with sizes below 0.1 λ (where λ is the light wavelength) the scattered light is highly polarised with an intensity proportional to λ^{-4} . For particles with sizes above 10 λ the scattered light is white and unpolarized. For intermediate sizes partial polarization occurs and the colours are unsaturated. As the scattering function is so complex, uniform sized dust can even produce red scattered light and volcanic dust in the stratosphere has been known to produce a blue moon.

The mass of moon smoke required to produce a TLP is of the order of 1 to 20 kg per sq. km. of lunar surface. Mills suggests that this could be blown above the moon's surface by episodic releases of gases which have accumulated in fissures and faults in the lunar rocks. These releases are triggered by tidal forces and coincide with moon quakes. The smoke quickly falls back to the surface thus explaining the transitory nature of the phenomena.

Now this mechanism could solve the TLP problem for the sunlit regions of the lunar surface but what about the TLP's on the dark side of the moon? If they exist (and there is some doubt) Mills considers that triboelectric discharges provide the only reasonable explanation. Gases expanding from subsurface regions blow away a cloud of dust which becomes charged by interparticle friction and undergoes charge separation. This cloud discharges when the product of the pressure and the mean dust separation distance reaches about 1,000 dyn cm⁻¹ and this condition is probably reached a few metres above the surface. The red colour is consistent with the gas being hydrogen.

Mills concludes that the triboelectric lightning discharges in gas-borne dust clouds can explain some TLP's especially those on the dark side of the moon. A more probable explanation requires less energy and less gas and simply relies on light being scattered by temporary clouds of moon smoke. □

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100 years ago

Mr. R. L. Jack, the Government Geologist of Queensland, has been carrying out his survey operations under difficulties unknown to home geologists. While he and his party were pursuing their explorations in the north of York Peninsula they were attacked by a band of natives, Mr. Jack receiving a spear in the neck, which had to be cut out. Fortunately the wound, though troublesome, is not likely to be attended with any serious or permanent results. North of Temple Bay Mr. Jack came upon a hitherto unknown large river, which he has named the "Macmillan."

The *Daily News* gives some account of a recent lecture by Prof. Palmieri on earthquakes. Prof. Palmieri went on to say that earthquakes have no doubt shorter or longer periods of preparation. The earth is never perfectly quiet for some time before and after a great shock, but gradually sinks into repose or increases in agitation. The Professor believes that, by registering the slight preliminary tremblings and noticing their increase or decrease it would be possible to foretell an earthquake about three days in advance, just as tempests are now foretold. If a connected system of seismographic stations were to be organised — the different stations communicating with each other by telegraph — it would be quite possible, in most cases, to issue warnings to the threatened district in time. The seismographic stations should be erected by the different Governments in quiet places where the ground was not liable to be shaken by heavy railway trains. From *Nature* 22, 17 June, 1880.

Planetary nebulae

from James B. Kaler

THEORIES of the formation of planetary nebulae are supported by the important discoveries of Hazard *et al.* reported in this issue of *Nature*.

During a large portion of their lives, stars lose mass back to interstellar space. In the final episode of mass loss, a highly evolved red giant star expels most if not all of its remaining hydrogen envelope as an expanding shell of gas. As the shell expands into space, it eventually is illuminated by the remaining core of the star, which becomes very hot. We then see the phenomenon as a planetary nebula, a shell or ring of gas surrounding a hot blue nuclear star which is contracting to its ultimate state as a degenerate white dwarf.

There are about 1,000 of these beautiful objects known, and perhaps 20,000 in the Galaxy at any given time. They are ephemeral, with lifetimes of perhaps 30,000 years, and come in a startling array of varieties. The nebulae are heated by photoionization from ultraviolet light provided by the central star. The spectra of the nebulae are dominated by recombination lines of hydrogen and helium, and by collisionally excited forbidden lines of various ionization stages of oxygen, nitrogen and neon. The observed radii range from only a few hundredths of a parsec to more than 0.5 pc, with the electron densities concomitantly varying from 10^6 cm^{-3} to less than 10^2 cm^{-3} .

The central stars display an equally wide variety, with effective black-body temperatures ranging from as low as 25,000 K (the minimum required to photoionize the nebula) up to perhaps 200,000 K,

making them as a class the hottest known stars. The properties of the stars and nebulae are correlated with one another, with the largest nebulae generally having the hottest central stars. The correlation is caused by the evolution of the star during the expansion of the nebula (O'Dell *Astrophys. J.* 183, 67; 1963; Abell *Astrophys. J.* 144, 259; 1966; Seaton *M.N.R.A.S.* 132, 113; 1966). However, a detailed explanation is complicated by the fact that the observed nebulae are produced by a large range in initial stellar mass (from about one to six times that of the sun), which results in a large and uncertain range of core masses upon which the individual evolution on the log luminosity (L) — log temperature (T) plane is dependent. (Paczynski *Acta Astronomica* 21, 417; 1971; Kaler *Astrophys. J.* 237; in the press.)

Since the mechanisms of spectral line formation are well understood, the abundance ratios of the lighter elements can be found rather directly from the relative line fluxes. About 100 nebulae have been so analyzed by a variety of people. The compositions of the nebulae reflect the state of the interstellar gas at the time and place of the origin of the star, but they also display effects of nuclear processing of the nebular gas in the original star before the ejection of the shell (Peimbert *IAU Symposium* 76, 215; 1977; Kaler *Astrophys. J.* 228; 163; 1979). In particular, many nebulae are considerably enriched in helium and nitrogen. The planetaries thus provide an important probe into the inner workings of stars, and observation of them can be used to test theories of the late stages of stellar evolution.

The enrichment of He and N in the planetary shell can be explained by a series

of "dredge-up" events that carry mass processed through the nuclear burning zones of the star upwards into the hydrogen envelope before it is lifted off to produce the nebula (Iben *Astrophys. J.* 140, 1631; 1964; 196, 525; 1975; 208, 165; 1976; Perinotto & Renzini *Astronomical Uses of the Space Telescope* (eds. Machetto Pacini & Tanenghi) 147, ESO, Geneva; 1978). The first of these processes occurs as the star ascends the giant branch for the first time after the exhaustion of its hydrogen core. A convection zone in the hydrogen envelope pushes into the now inactive hydrogen-burning shell that surrounds the helium core, lifting away nitrogen that has been created from carbon during CNO burning. The second episode takes place during the second ascent of the giant branch for stars larger than about $3 M_{\odot}$ where the star now has an inert carbon-oxygen core which is surrounded by concentric helium and hydrogen burning shells. This time the convection zone sweeps into the helium core produced by the earlier burning of the hydrogen shell, and not only carries away more nitrogen, but large quantities of helium as well. To this point, the theoretical correlation between the He/H and N/O ratios in the hydrogen envelope agrees well with those observed for planetary nebulae (Kaler, Iben & Becker *Astrophys. J. Lett.* 224, L63; 1978.) The final event occurs during the thermal pulsing stage on the second ascent, when convection penetrates into a region processed by the helium-burning shell, which further alters the composition of the envelope.

Renzini (*Fourth European Mtg. in Astronomy: Stars and Stellar Systems*, ed. Westerlund, 155, Reidel, Dordrecht) has placed these abundance variations within the framework of the theoretical evolution of the star on the log L -log T plane. Because of different rates of evolution, high mass stars, for which the largest helium and nitrogen enrichments should be found, are expected to appear as high temperature, low luminosity planetary nuclei. Stars of this kind are seen at the centers of the largest nebulae.

The above theories attempt to describe the conventional planetary in which processed material may be mixed into the hydrogen envelope, before it is expelled to form the nebula. But the paper by Hazard *et al.* in this issue of *Nature* adds something entirely new and of great significance. They have discovered knots of gas near the central star of the planetary Abell 30, in which the He/H ratio is at least 20 times its normal value. They thus demonstrate that not only did the formation of Abell 30 use the entire residual hydrogen envelope of the star, but that the star is in fact ejecting some of its current helium-rich envelope and that the nuclear star is extremely hot. Hazard *et al.*'s observations agree with Renzini's hypothesis, and our current notions of the development of the planetary nebulae. □

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Isolating colour vision mechanisms with hue substitution

from R. W. Bowen, J. Pokorny
and V. C. Smith

In a review of new psychophysical techniques for isolating color-opponent neural mechanisms in human vision, J. D. Mollon¹ expresses the opinion that hue substitution² — a form of pure chromaticity modulation in which a chromatic field is briefly exchanged for a white of equal luminance — is not likely to activate selectively chromatic visual channels since it may generate receptor transients detectable by 'luminance' channels. At issue is whether data obtained with hue substitution reflects functional isolation of chromatic channels or not.

Studies of visual temporal processing with hue substitution stimuli have shown that visual latency³, two-pulse discrimination⁴ and duration thresholds⁵ follow a wavelength function resembling trichromatic saturation discrimination: slowest temporal response at 570 nm and brisker temporal responses at the spectral extremes, suggesting that the speed of the temporal response reflects activity in red-green and blue-yellow opponent-color mechanisms.⁶⁻⁸

Mollon is among those⁹⁻¹² who have suggested that the 'violet-sensitive' receptor does not contribute to luminance channels. If we accept this postulate, receptor transients in '565-nm' and '535-nm' cones cannot explain the wavelength dependence of hue substitution data. Consider hue substitution from white to an equiluminant spectral yellow or violet where these lights are collinear on a tritanopic isochromatic line. Such stimuli have equal quantal catch for the '565-nm' and '535-nm' receptors; therefore receptor transients cannot occur at stimulus presentation. Receptor transients predict only a 'tritanopic' wave-length dependence; in fact, such dependence has been found for two-pulse discrimination of brief chromatic stimuli.⁴ However, to ascribe this function to receptor transients demands the additional restraint that normalization of '565-nm' cone sensitivities is identical for both chromatic and luminance channels, a hypothesis difficult to reconcile with estimates of cone spectral sensitivities.^{10,11,13,14}

The issue is addressed directly in the case of duration thresholds⁵: just-detectable hue substitution stimuli are always perceived as chromatic, a phenomenon which cannot be mediated by receptor transients in luminance channels. Further, the wavelength

dependence of temporal processing in hue substitution is identical to that observed with an independent method of measuring chromatic channel function (discriminative reaction time).¹⁵ Finally, metacontrast masking¹⁶ and temporal brightness enhancement¹⁷ are abolished with hue substitution stimuli: these phenomena require physical luminance transients.

Transient receptor responses could in principle arise from pure chromaticity modulation and might be important in some situations (e.g., high luminance levels). However, there is strong evidence that chromatic neural mechanisms dominate observed effects on visual function in the hue substitution literature cited here. □

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J. D. Mollon replies

FOR validation of hue-substitution techniques, Bowen, Pokorny and Smith (their 2nd paragraph) turn primarily to the finding that measures such as reaction time vary with wavelength in hue-substitution experiments in the same way as does threshold in experiments on saturation discrimination. But this result is equally to be expected if the speed of response depends on the magnitude of transient responses generated by the 565-nm and 535-nm cones at the moment of substitution. For saturation depends ultimately on the degree to which the ratios of absorptions in different classes of cone differ from the ratios produced by white light. At a spectral locus close to 570 nm is a tritanopic neutral point (i.e. there is a yellowish monochromatic light that those who lack the short-wavelength receptors confuse with the white used in the experiment). I think we share a common theory of what this means: when this monochromatic light and the white are equated in luminance, the yellow produces exactly the same absorptions in the long- and middle-wavelength cones as

does the white. When the monochromatic light is substituted for the white there will be no change in the signals from these cones. Response to a substituted 570-nm field will be slow, either because it is mediated only by short-wavelength receptors or — if 570 nm does not exactly coincide with the tritanopic neutral point — because the residual transients in the long- and middle-wavelength cones are very small. As the wavelength of the substituted field increasingly diverges from 570 nm, the receptor transients must increase, since the ratio of absorption in the long-vs. the middle-wavelength cones must significantly differ from that produced by white light. (The short-wavelength tritanopic neutral point has not been examined in hue-substitution experiments.)

This argument does not require us to know the ratio of absorption that white light produces in the long- and middle-wavelength cones and there is no need to suppose that this ratio (or the ratio of any subsequent signals) is unity; we need allow only that these ratios remain constant (or, in practice, almost constant) across the substitution to 570 nm. Bowen, Pokorny and Smith's reference to 'normalization' (their 3rd paragraph) is obscure but I would diffidently suggest that they are confounding (a) the wavelength at which the two classes of cone deliver equal signals to a putative luminance channel and (b) the wavelength (c. 570 nm) that delivers the same ratio of signals as does white light. If wavelength (a) is indeed remote from (b), if, say, it is 500 nm, then the ratio of signals produced by white light must be very different from unity and large transients must occur at the passage to wavelength (a).

Reference 5 does not contain formal data on perceived hue, and, being concerned only with liminal stimuli, it cannot validate the entire hue-substitution technique. To say discriminative reaction time (ref. 15) depends on saturation is to say it depends on the extent to which the absorptions produced in the several classes of cones differ from those produced by white light; the magnitudes of receptor transients depend on these very differences. In the metacontrast experiment (ref. 16) the dependent variable was the degree of phenomenal darkening of the target; when the masking stimuli were 'equiluminant', receptor transients would be of opposite sign for different cone classes and hence the net effect on target brightness cannot be predicted. □

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ARTICLES

Cytoplasmic free calcium and amoeboid movement

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*Measurements with the Ca^{2+} -sensitive photoprotein aequorin show that locomotion in the amoeba *Chaos carolinense* occurs without changes in the aequorin signal and that not more than 0.025% of the cytoplasm can exist at the micromolar threshold concentration for contraction. The results do not support the hypothesis that cytoplasmic streaming is under the control of changes in the cytoplasmic free Ca^{2+} concentration.*

It is widely believed that the intracellular control of cell motility may involve modulation of actomyosin activity by elevated cytoplasmic free Ca^{2+} concentrations. Many non-muscle cells have been shown through insult with Ca^{2+} buffers to have a threshold for contraction of approximately micromolar free Ca^{2+} , and studies on Ca^{2+} -activation of non-muscle myosins have revealed Ca^{2+} -sensitizing factors associated with both myosin and actin. Elevated Ca^{2+} concentrations are also thought to play a part in cytoplasmic consistency (sol-gel) changes¹⁻⁶. In view of the central controlling role attributed to Ca^{2+} it is surprising that investigations of physiological Ca^{2+} changes accompanying motile events in the normal cell have given equivocal results, with Ca^{2+} concentrations being apparently invariant or well below threshold levels⁶⁻¹¹. In *Chaos* a micromolar Ca^{2+} threshold for contraction has been shown using Ca^{2+} -EGTA buffers in conjunction with naked, 'flaring' cells¹², naked cytoplasmic strands^{12,13}, injections into intact amoebae¹⁴ and ultrastructural studies^{15,16}, providing extensive support for the concept of control by Ca^{2+} . It is shown here by means of calibrated aequorin measurements that not more than 0.025% of the cytoplasm of a locomoting amoeba can exist at the micromolar threshold for contraction and that changes in the pattern of cytoplasmic streaming are not accompanied by changes in the aequorin signal.

Calibration of the aequorin signal

Aequorin is a photoprotein which needs only to bind Ca^{2+} in order to emit light, being independent of oxygen, ATP or other cofactors¹⁷, and which has been widely used as an indicator of cytoplasmic free Ca^{2+} concentrations^{18,19}. The photomultiplier signal from an aequorin-injected amoeba, typically between 10 and 100 photoelectrons per second (background 5 s^{-1}), was expressed as a fraction of the total aequorin content of the cell, as measured by recording the light emitted during killing of the cell, to give a value for the rate constant of aequorin consumption *in vivo* (Fig. 1). A value for the cytoplasmic free Ca^{2+} concentration was derived from rate constants determined *in vitro* over a range of free calcium concentrations set by Ca^{2+} -EGTA buffers (Fig. 2). Between 10^{-7} M and 10^{-6} M Ca^{2+} the rate constant rises by three orders of magnitude giving excellent sensitivity to small changes in Ca^{2+} concentration in the range of interest. Care was taken to ensure that the ionic conditions *in vitro* mimicked those in the amoeba groundplasm^{20,21}, since the sensitivity of aequorin to Ca^{2+} is depressed by increasing ionic

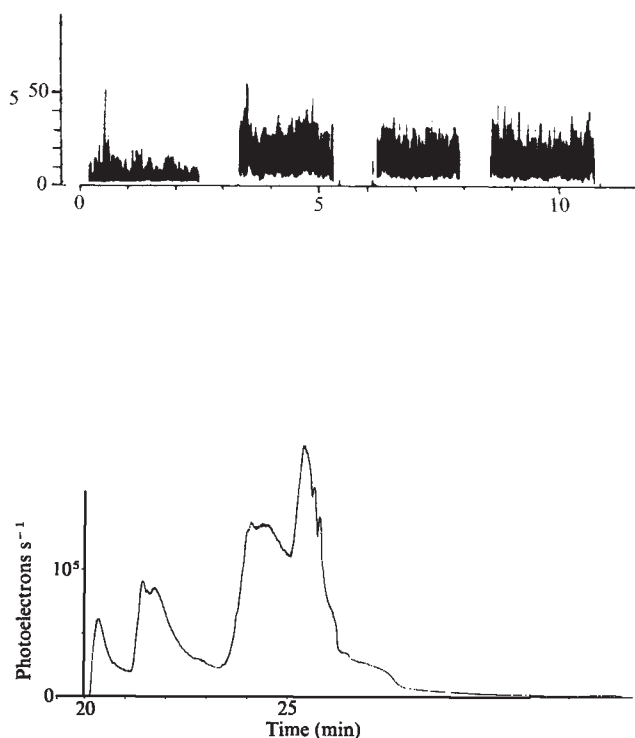
strength and free Mg^{2+} (refs 22, 23). The free Mg^{2+} concentration *in vitro* was equal to the total Mg^{2+} content of whole cells minus crystalline bodies^{20,24} and is probably greater than the actual cytoplasmic free Mg^{2+} , resulting in an overestimate of free Ca^{2+} . This method of external calibration is valid only if the sensitivity of aequorin to Ca^{2+} is the same *in vivo* as *in vitro* and, although aequorin is unaffected by the presence of EGTA²⁵, there is some evidence that it might be inhibited by unknown cytoplasmic factors²². This possibility was assessed by means of 'internal calibration' experiments^{22,26} in which rate constants were determined in amoebae injected with EGTA-buffers (Fig. 2 legend). There is no evidence for anomalous Ca^{2+} -sensitivity of aequorin *in vivo*. Calibration errors arising from self-absorption of light within the cell were not important since normal rate constants were recorded from locomoting amoebae which had previously been clarified by centrifugation and bisection. Calcium binding to EDTA injected as a contaminant of aequorin was negligible, the low cytoplasmic concentration, less than $5\text{ }\mu\text{M}$ EDTA, being entirely satisfied by Mg^{2+} . The cytoplasmic concentration of aequorin, approximately $1\text{ }\mu\text{M}$ (ref. 10) will bind approximately 16% of free 10^{-7} M Ca^{2+} (ref. 27), a small displacement which was presumably replaced from millimolar intracellular Ca^{2+} stores^{20,24}. Injected amoebae recovered normal locomotion within 15 min, suggesting that the injected solution had no persistent traumatic effects. Location of aequorin in the groundplasm compartment was demonstrated by freezing an aequorin-laden cell, previously stratified by centrifugation, under an image-intensifier microscope whereupon most of the light originated from the centripetal half, most of the aequorin being excluded along with groundplasm from the organelle-rich centrifugal end. A similar approach showed that aequorin mixed throughout the groundplasm of normal cells¹⁰. Other precautions to satisfy the protocol proposed by Blinks *et al.*²⁶ are described in Fig. 1.

Aequorin signal and motility

The mean aequorin rate constant (k) *in vivo* measured on cells in various motile states, predominantly radiate (that is, a rounded and unattached cell with several radiating spindly pseudopodia), was $1.82 \times 10^{-6} \pm \text{s.d. } 1.58 \times 10^{-6}\text{ s}^{-1}$ ($n=32$). Hence $\log k$ mean was -5.74 , (range $\pm \text{s.d. } -5.47$ to -6.6), giving a mean free calcium concentration, at 5 mM free Mg^{2+} , of $8 \times 10^{-8}\text{ M}$ ($1.5 \times 10^{-7}\text{ M} + \text{s.d.}, 2 \times 10^{-8}\text{ M} - \text{s.d.}$), falling to $5 \times 10^{-8}\text{ M}$ in 0.5 mM free Mg^{2+} . Since the actual

Fig. 1 Chart record of an experiment to determine the rate constant of aequorin consumption in an amoeba showing photomultiplier anode current expressed as photoelectrons s^{-1} against time in minutes. *Upper trace:* 0–3 min background current of 5 photoelectrons s^{-1} ; 3–11 min signal from a single aequorin-injected amoeba, 10 photoelectrons s^{-1} , superimposed on background. The trace was blanked out during observation and photography. *Lower trace:* lysis of the same amoeba beginning 8 min after adding a minute quantity of solid SDS to the culture medium (1 ml), accompanied by discharge of the aequorin. An approximately 10-fold higher concentration of SDS added to aequorin in a Ca^{2+} buffer did not affect the light yield as judged by the agreement between the predicted and observed areas under the discharge curve. The peaks on this curve represent a wave of lysis spreading across the cell. The cell surface is extraordinarily resistant to lysis, probably by virtue of a thick glycocalyx, and the high extracellular Ca^{2+} upon entry into the cell at a lytic focus causes a local gelation and pinching-off of that region by a 'surface precipitation reaction'⁴⁰; the peaks do not represent lysis of different intracellular compartments. The area under this discharge curve was equivalent to 3.3×10^7 photoelectrons so that the rate constant for aequorin consumption, k , in the intact amoeba prior to lysis was $3 \times 10^{-7} s^{-1}$. Hence $\log k$ was -6.5 , corresponding to a cytoplasmic free Ca^{2+} concentration of $4 \times 10^{-8} M$ (see Fig. 2). Correction of the total photoelectron count for photocathode quantum efficiency (12%) geometric counting efficiency (2.5%) and aequorin quantum yield (23%) gave a total aequorin content of 4.7×10^{10} molecules and an aequorin concentration in a cell of 40 nl volume of $2 \mu M$. Aequorin: a saturated ammonium sulphate precipitate of aequorin purified by ion-exchange chromatography⁴¹ was desalted by centrifugation, dialysis against Chelex (Biorad) decalcified 5 M potassium acetate followed by gel filtration on Sephadex G-25 using 10 mM ammonium formate $1 \mu M$ EDTA as eluent²⁵, and freeze dried. A microscopic speck of freeze-dried protein was dissolved in 100 nl of injection buffer (30 mM potassium acetate + $1 \mu M$ EDTA) held in a 3 mm length of microdialysis tube (Biofiber 50, Biorad) and dialysed for 1–3 h against several changes of buffer to remove EDTA contaminating the freeze-dried aequorin. The EDTA concentration in the dialysed solution was shown by back-titration to be less than $50 \mu M$. Amoebae were injected to ~5% of their volume and usually recovered normal locomotion in 15 min. All experiments used aliquots from a single freeze-dried sample of aequorin to minimize errors from possible differences between preparations⁴⁶. All *in vivo* measurements were made within 12 h of injection and all *in vitro* measurements before the signal had decayed 20% in order to minimize errors caused by significant consumption of aequorin.

For equipment details see ref. 10. Temperature $20 \pm \frac{1}{2} ^\circ C$.



groundplasm free Mg^{2+} was probably between these limits the mean free Ca^{2+} concentration was between 50 and 80 nM. The variability in rate constant was probably due to the number of rounded, quiescent or radiate cells in the sample. Such cells often showed a slow rise and fall in the aequorin signal over a period of approximately 30 min, with Ca^{2+} rising from around 7×10^{-8} to $2 \times 10^{-7} M$, without any discernible motile activity. At their peak, these signals usually showed spiky fluctuations with a fast rise time which were not due to shot noise (compare Fig. 3b and c) and which were not abolished with external 10 mM EGTA or La^{3+} . Rounded cells (Fig. 3b) usually showed larger signals than locomoting cells and resumption of cytoplasmic streaming was accompanied by a fall in signal to levels similar to that in Fig. 3a, a change which cannot be attributed to counting geometry, which would operate in the reverse sense. The significance of these observations on rounded cells, whether quiescent or radiate, is not known, but the calcium changes did not correlate with changes in shape or streaming activity.

The aequorin signal from actively locomoting cells, whether polypodial or monopodial, showed little fluctuation above that due to shot noise and was usually lower than in quiescent cells (Fig. 3a). Signals recorded over periods of hours on more than 50 cells did not reveal any minute-to-minute changes which could be correlated with the initiation, cessation or reversal of cytoplasmic streaming or to changes in adhesion or cell shape. Even dramatic spikes such as that in Fig. 3b, which were infrequent (1 or 2 per hour) did not appear to influence shape or movement. It is impossible to escape the conclusion that changes in the pattern of cytoplasmic streaming in the pseudopodia of a locomoting cell are not accompanied by changes in the aequorin signal and therefore in the mean cytoplasmic free Ca^{2+} concentration.

It could be argued that failure to detect Ca^{2+} changes during locomotion might merely be due to inadequate sensitivity in the technique. However, it is apparent from Fig. 2 that raising 1% of a cell's volume to the $10^{-6} M$ Ca^{2+} threshold for contraction¹⁴ would give $\log k = -4.8$ which, for a typical cell (for example, Fig. 1) would generate a signal of 520 photoelectrons s^{-1} , considerably greater than the observed signal of $10 s^{-1}$. Therefore the technique possesses excellent sensitivity, being capable of measuring sustained threshold concentrations of Ca^{2+} in small zones of the amoeba. However, it is conceivable that transient rises in free Ca^{2+} , too brief to be resolved with the present technique, might be responsible for triggering a slow-decaying Ca^{2+} -independent active state of the contractile proteins. Thus a zone of micromolar Ca^{2+} occupying 1% of cell volume and generating 520 photoelectrons s^{-1} would, if it persisted for 100 ms produce a spike in the signal which, because of limitations in the response time of the instrumentation, would be impossible to resolve amongst the Cerenkov background. In the analysis that follows it has been assumed that the elevated Ca^{2+} concentration has to be maintained in order to sustain contraction.

Zones of micromolar calcium?

The above estimate of a mean cytoplasmic free Ca^{2+} concentration of 50 to 80 nM was based on the assumption that Ca^{2+} was distributed homogeneously throughout the groundplasm, but it is conceivable that a fraction of the signal arises from local zones at the micromolar threshold for

contraction described by Taylor¹⁴ (free Ca^{2+} values in Taylor's buffers have been recalculated; compare Fig. 2 legend). Thus, in 10^{-6} M Ca^{2+} $\log k = -2.8$ (Fig. 2) so the *in vivo* mean $\log k$ (-5.8) could have been generated by a zone of micromolar Ca^{2+} influencing 0.1% of the aequorin content of the cell. However, if allowance is made for light contributed from the remaining 99.9% of the aequorin, which must generate at least 10% of the signal since even in the complete absence of Ca^{2+} $\log k$ is -6.8 due to the calcium-independent light²⁸, the zone falls to 0.09%. If a more realistic value for the free Ca^{2+} concentration in the bulk of the cytoplasm is used, for example 4×10^{-8} M, then the micromolar zone cannot exceed 0.07% cell volume. If the actual cytoplasmic free Mg^{2+} concentration is less than 5 mM, as it most probably is, then further restriction of the micromolar zone occurs. Thus, at 0.5 mM Mg^{2+} the *in vivo* rate constant would be generated by 5×10^{-8} M Ca^{2+} , probably close to the free Ca^{2+} concentration in the bulk of the cytoplasm, whereupon the possible extent of the micromolar zone falls to zero. Furthermore, active locomoting cells showed lower rate constants than the mean (for example Fig. 3a, $\log k = -6.04$) and the scope for micromolar zones becomes vanishingly small, that is, 0.025% at 5 mM Mg^{2+} ; 0% at 0.5 mM Mg^{2+} (bulk free Ca^{2+} assumed to be 4×10^{-8} M).

Image intensifier observations

In the more recent of their two image intensifier studies on aequorin-laden amoebae Taylor *et al.*^{8,9} report luminescence arising spontaneously in the tail of the cell. Although precise calibration was not attempted, the free Ca^{2+} concentration was less than micromolar as judged by the signal from injecting 10^{-6} M Ca^{2+} buffer⁹. In an independent study using a similar intensifier¹⁰ I failed to detect any signal from locomoting, aequorin-laden cells. This difference cannot be attributed to the amoebae since our aequorin rate constants agree [$\log k$ -5.8 and -5.6 (D. L. Taylor, personal communication)] and was probably caused by insensitive photographic recording of the intensifier output phosphor, which would raise the detection limit. Figure 4 gives an estimate of the intensifier detection limit: it was possible to detect compact zones of micromolar and submicromolar Ca^{2+} in the cell. The zone of 10^{-6} M Ca^{2+} occupying 0.05% of cell volume represents the upper limit imposed by the rate constant data and would have been detectable above the randomly distributed background. Similarly the image from 1% at 4×10^{-7} M Ca^{2+} , again at the limit imposed by the rate constant data (assuming 5 mM Mg^{2+} and 40 nM Ca^{2+} in bulk of cell), would have been recognizable. The absence of a detectable signal from *Chaos* indicates that it is unlikely that micromolar zones of elevated Ca^{2+} occurred. A constraint to this argument against the existence of micromolar zones is that this analysis applies only to compact, spherical Ca^{2+} zones. An elevated Ca^{2+} zone in which the photoelectron signal is dispersed over a large image area would not be detected above background.

Does free Ca^{2+} control motility?

The most obvious interpretation of these data is that the cytoplasmic free Ca^{2+} concentration is uniformly 8×10^{-8} M (given 5 mM free Mg^{2+}). Such a conclusion would be consistent with the constant level of the aequorin signal during motility changes, the vanishingly small scope for zones of elevated Ca^{2+} and with the failure to image zones of elevated Ca^{2+} despite adequate intensifier detection limits. However, this conclusion conflicts with the requirement for micromolar calcium for contraction in EGTA buffers¹⁴ and as the site(s) of force generation in *Chaos* is not known^{29,30}, arguments can be raised in support of the concept of small zones of threshold Ca^{2+} , occupying not more than 0.025% of the cell, being responsible for triggering motile activity. One such argument concerns the constant level of the aequorin signal and the

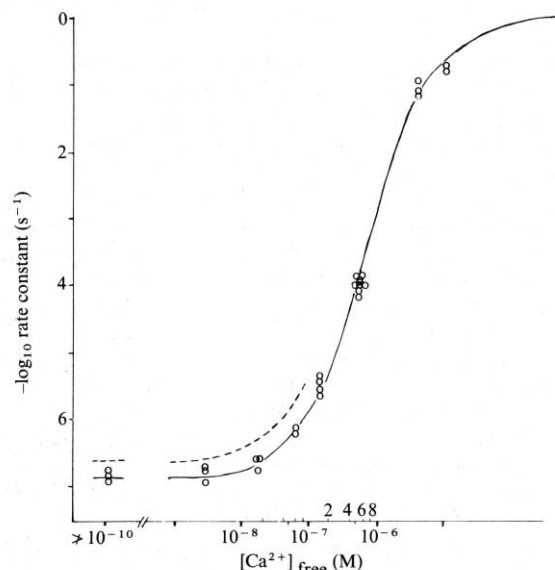


Fig. 2 Dependence of the rate constant for aequorin consumption (k) upon the free calcium concentration *in vitro*. Double log plot. Points on the curve are individual determinations using quantities of aequorin similar to those injected into single amoebae, so that instrumental errors are comparable. Rate constants were determined by expressing the light flux in the buffer as a fraction of the total light recorded during discharge of the remaining active aequorin following addition of calcium acetate. Composition of buffer (mM): KCl 30, free Mg^{2+} 5, EGTA 20, MOPS 10, KOH to pH 6.8. The broken line was extrapolated from the data of Allen *et al.*²⁵ for $[\text{Mg}^{2+}]_{\text{free}} = 0.5$ mM. Free calcium concentrations were calculated by successive approximation⁴² allowing for Mg-EGTA using Schwarzenbach's apparent association constants appropriate for pH 6.8⁴³ ($\text{Ca-EGTA } k_{\text{app}} = 1.95 \times 10^6$; $\text{Mg-EGTA } k_{\text{app}} = 23.99$). For the sake of comparability the free calcium concentrations in the series of EGTA buffers used by Taylor *et al.*¹²⁻¹⁶ have been recalculated using these association constants. As a result their 'physiological threshold for contraction' of 7×10^{-7} M becomes 1.1×10^{-6} M. The Ca^{2+} -sensitivity of aequorin in the cytoplasm was tested by internal calibration experiments in which a Ca^{2+} -buffer (mM: KCl 30, EGTA 20, MOPS 10, pH 6.8) was injected into an aequorin-laden amoeba to ~5% of the cell volume while recording the light signal. Injection of 10^{-5} M free Ca^{2+} gave a peak signal which decayed rapidly to a low level. Subsequent lysis of the cell with SDS then elicited a second, much larger release of light. The ratio between the areas under the first and second curves was approximately 20 suggesting that, perhaps, the injected buffer had mixed with only ~5% of the cytoplasm. The half life of the decay in light intensity immediately after the injection, between 2.5 and 8 s, was comparable with the half life expected from the rate constant *in vitro* (4 s). Furthermore the rate constant for aequorin consumption measured on the first peak was only 0.3 log unit less than the value obtained *in vitro*. While interpretation of these experiments is complicated by a lack of direct evidence for poor mixing (an image intensifier was not available), it seems unlikely that the explanation for the low peak light intensity following buffer injection resides in an inhibition of aequorin *in vivo* since it is then necessary to postulate a rapid sequestration of Ca^{2+} in order to explain the rapid decay in light intensity. Poor mixing of Ca^{2+} -containing solutions, which appear to elicit local gelation and a 'surface precipitation reaction'⁴⁰, is also seen in the slow-spreading discharge of aequorin in a lysing cell (Fig. 1). Exact agreement between rate constants and half-lives *in vivo* and *in vitro* should perhaps not be expected in view of the possibility of a slow spread of buffer into fresh aequorin-laden cytoplasm (prolonging half life), sequestration of Ca^{2+} (shortening half life), pH shifts affecting EGTA and uncertainty of the free Mg^{2+} *in vivo*. Light measurements following injection of 10^{-8} M Ca^{2+} buffer had to be made after withdrawal of the micropipette to eliminate the relatively large signal arising around the site of impalement⁹. $\log k$ after injection was -6.5 compared with -6.8 *in vitro*. This difference may be due to Ca^{2+} release from stores, a lower free Mg^{2+} *in vivo*, residual micropipette injury, or perhaps to poor mixing, although low- Ca^{2+} buffers appear microscopically to mix readily with the cytoplasm.

possibility that a rising signal from a zone starting to contract might be exactly compensated by a simultaneous loss of signal from a zone undergoing relaxation, giving a false impression over the cell as a whole of a constant Ca^{2+} concentration. However, the approximately cube-law relationship between signal and free Ca^{2+} (Fig. 2) suggests that the probability of the product of the volume and Ca^{2+} concentration of both zones being so balanced as to generate a constant signal would be low. It is more likely that a constant signal indicates a constant free Ca^{2+} concentration. However, it is not possible to rule out the Ca^{2+} concentration changes with absolute certainty and, by assuming that the bulk of the cell is at $4 \times 10^{-8} \text{ M}$ rather than $8 \times 10^{-8} \text{ M}$ Ca^{2+} (5 mM Mg^{2+}), there is sufficient surplus signal to encompass a zone of 10^{-6} M Ca^{2+} occupying 0.025% of the cell volume. Two arguments suggest that it is unlikely that a volume of cytoplasm of this size could be responsible for generating the motive force. If an actively locomoting cell moves through its own length in 3 min cycling all the cytoplasm through the micromolar zone, then the mean residence time of cytoplasm in the zone would be 50 ms, and the appearance given by such brief contraction in a highly localized area of the cell would be inconsistent with the smooth-flowing appearance of normal cytoplasmic streaming. Furthermore injection of 1% of cell volume with 10^{-6} M free Ca^{2+} in an EGTA buffer influenced a volume of cytoplasm approximately ten times larger (P.H.C., unpublished), but only induced contraction in a zone ~ 50 to $100 \mu\text{m}$ in diameter¹⁴, considerably smaller than the normal endoplasmic stream which can be $1,000 \mu\text{m}$ long. Therefore a micromolar calcium zone of 0.025% cell volume, 40 times smaller than in the injection experiment, would be quantitatively inadequate for inducing the observed cytoplasmic flows. It follows that models of cytoplasmic streaming based upon contractions or gelation changes induced by a stationary zone of micromolar Ca^{2+} through which the cytoplasm flows, such as might be envisaged in the recruitment and fountain zones, are not

tenable. In addition, such zones would be relatively compact and would have been detected by image intensification (Fig. 4 and ref. 9).

The rate constant and intensifier data are perhaps compatible with a contraction-hydraulic model involving Ca^{2+} -induced contraction of a thin sub-plasmalemmal cortex in the rear of the cell. A micromolar Ca^{2+} zone of 0.025% cell volume would be adequate to invest the internal surface of half the plasmalemmal area to a depth of $0.5 \mu\text{m}$, comparable in extent to the layer of filamentous cytoplasm recently described in the rear of the amoeba^{31,32}. Problems of residence time do not arise and the elevated Ca^{2+} could be derived from the transplasmalemmal currents described by Nuccitelli *et al.*³³, which have been implicated in the tail luminescence detected by Taylor *et al.*⁹. It is not possible on the available evidence to determine whether Ca^{2+} -induced contractile activity in the sub-plasmalemmal zone contributes to cytoplasmic streaming. However, it is conceivable that local micromorphological surface movements could be markedly influenced by Ca^{2+} -induced contraction or gelation changes in the thin felt of microfilaments. A combination of image intensification and the extracellular current probe³³ should help to resolve this question.

In smaller cells such as *Acanthamoeba* with a volume of 0.01% that of *Chaos*, or in the 'ruffling membrane' of fibroblasts, it is not difficult to envisage a role for transplasmalemmal Ca^{2+} currents in inducing contractile activity of a sub-plasmalemmal network of microfilaments. Indeed, in view of the larger surface-to-volume ratio of small cells, cortical contractile activity might make an appreciable contribution to the total locomotory effort, but experimental evidence for a direct role for Ca^{2+} in initiating sub-plasmalemmal contraction will be difficult to obtain.

Whatever the contribution of subplasmalemmal contractions to streaming in intact *Chaos* they cannot be responsible for the motile activity of naked cells held in capillaries^{29,30} or

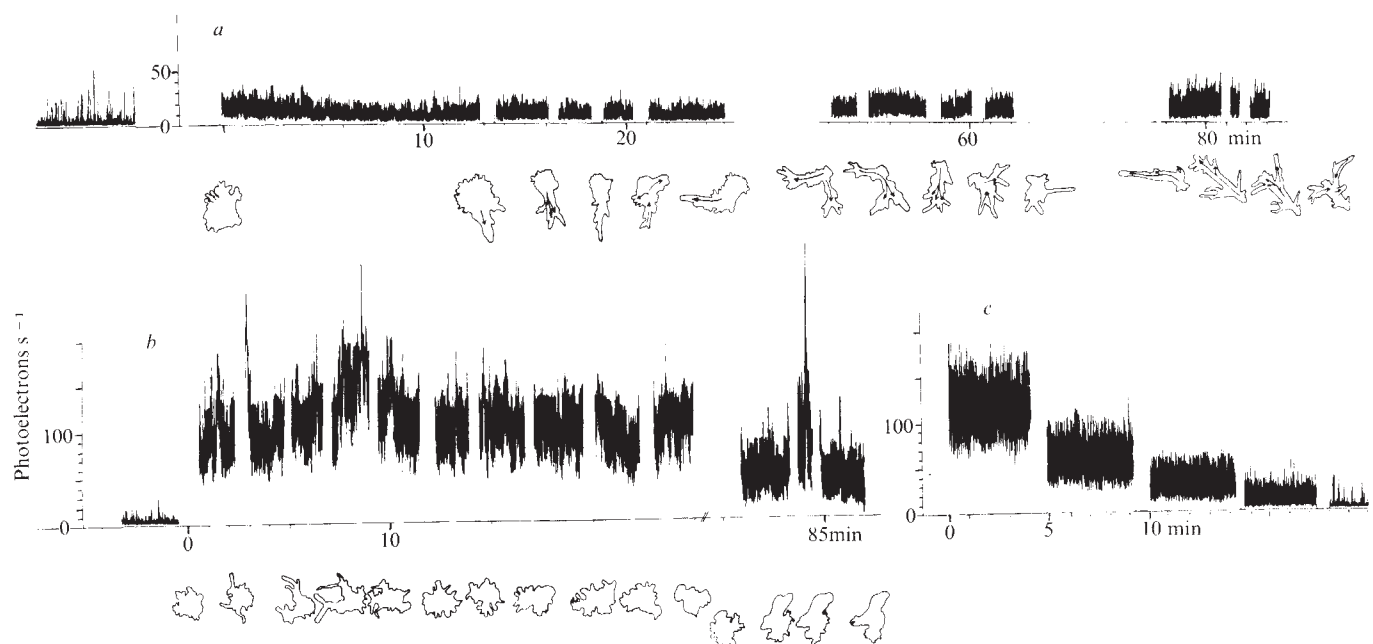


Fig. 3 Chart recordings of aequorin signals from two representative amoebae, with cell outlines traced from photographs and the direction of cytoplasmic streams arrowed. (The relative positions of the outlines are not an indication of the distance covered by the cell between photographs.) *a*, Actively locomoting, polypodial amoeba. The signal ($\log k = -6.04$) remained more or less constant and showed no consistent changes with locomotory behaviour of the amoeba, a typical result. Between minutes 78 and 81 the cell moved through its own length, yet the signal was not discernibly different from the initial signal from the cell in a rounded, static condition. *b*, Rounded cell, occasionally extending spindly pseudopodia in a radiate-like fashion. No locomotion. The spikes and slower fluctuations are greater than variations due to shot noise (compare with *c*), and are found in rounded, radiate or quiescent cells but not in locomoting cells. The large spike at 84 min had no effect on cell shape or streaming. The source of the fluctuations is not known but they do not appear to correlate with the production of pseudopodia, nor are they dependent on external calcium. *c*, Shot noise at mean count rates of 130, 70, 30, 15 photoelectrons s^{-1} , followed by background (5 s^{-1}). All observations were made in Marshall's medium²¹ on amoeba maintained on mixed ciliates. Recording chambers, of glass, polycarbonate or photographic film-base were kept in darkness prior to use to eliminate phosphorescent background¹⁰. Amoebae were illuminated in red light during observation and photography.

exposed to EGTA buffers¹². Naked cells in capillaries continue to stream for up to an hour while naked cells bathed in EGTA buffers show motile activity which is apparently dependent on the free Ca^{2+} concentration, being immobile in 10^{-7} M, showing 'flare' streaming in 1.1×10^{-6} M and contracting radially in $\sim 3 \times 10^{-6}$ M (refs 6, 12). (The free Ca^{2+} levels have been recalculated for comparability with this study, see Fig. 2 legend). There is a serious discrepancy between these experiments and the aequorin measurements in that the concentration of free Ca^{2+} in the 'flare' solution, 1.1×10^{-6} M, is 14 times greater than the mean free Ca^{2+} in the cell, that is, 4 to 8×10^{-8} M depending on the free Mg^{2+} concentration. The cause of this discrepancy is not known, but it cannot be attributed to the absence of the plasmalemma since naked cells confined in quartz capillaries or under oil continue to stream^{29,30}. One possibility is that the inhibition of motility in 10^{-7} M Ca^{2+} buffer resulted from the presence of EGTA since birefringence and ultrastructural studies have demonstrated a rapid loss of microfilaments in buffers of higher free EGTA concentrations (5 to 25 mM)^{13,16}. Furthermore injection of a 5 mM EGTA buffer giving 1×10^{-7} M free Ca^{2+} , a physiological level, into intact amoebae caused a cessation of locomotion and a loss of distinction between ectoplasm and endoplasm¹⁴. While it is possible to explain the microfilament depolymerizing effects of high EGTA concentrations on the higher buffering capacity and hence upon the lower free Ca^{2+} levels achieved in the presence of Ca^{2+} leakage from organelles¹⁶, this does not explain why motility is lost in physiological levels of 10^{-7} M Ca^{2+} and restored only when free Ca^{2+} is raised to 14 times normal (1.1×10^{-6} M). It may be significant that the free EGTA concentration in the 10^{-6} M buffer was ~ 0.8 mM, markedly lower than in the 10^{-7} M buffer, and the possibility has to be recognized that free EGTA at concentrations of ~ 5 mM may not be compatible with normal motile behaviour, even at physiological free Ca^{2+} levels. The question therefore arises as to whether the Ca^{2+} threshold for contraction and gelation is, in the absence of EGTA, less than micromolar. If this is so, then the control concentrations would have to be considerably less than micromolar in order to comply with the aequorin data if appreciable volumes of cytoplasm are to be influenced. Thus zones occupying 1%, 5% and 10% of the cell volume cannot exceed free Ca^{2+} concentrations of 4×10^{-7} , 2.5×10^{-7} and 2×10^{-7} M respectively. Such zones, if compact, would have been detectable with the image intensifier (Fig. 4) suggesting that the putative submicromolar threshold must be closer still to the resting Ca^{2+} level, or smaller in extent. Condeelis *et al.*¹⁶ have made the interesting suggestion that microfilament depolymerization might be promoted in the normal cell by lowered Ca^{2+} concentrations. Indeed, the aequorin data would be entirely compatible with such a scheme since (see Fig. 2) if 10% of the cytoplasm were to undergo a reduction in Ca^{2+} from 8×10^{-8} M to 4×10^{-8} or 2×10^{-8} the fall in the signal would be 6% and 8% respectively, and would be undetectable in the noise. Further aequorin measurements, preferably on naked cells in capillaries where contributions to the signal from sub-plasmalemmal Ca^{2+} zones are eliminated, are obviously required to determine whether such subtle rises or falls in the free Ca^{2+} concentration are involved in motility control. At present, however, the involvement of free Ca^{2+} changes either side of the resting level must be regarded as uncertain.

The aequorin data are consistent with a constant level of cytoplasmic free Ca^{2+} , but this interpretation does not necessarily mean that the control mechanism is Ca^{2+} -independent or that Ca^{2+} binding to motile proteins is unimportant. On the contrary, the evidence of Taylor *et al.*¹²⁻¹⁴ has demonstrated the sensitivity of cytoplasm to Ca^{2+} from 10^{-6} to 10^{-3} M beyond reasonable doubt. So it is conceivable that control of streaming through a constant- Ca^{2+} mechanism may involve modulation of the sensitivity of the contractile proteins to Ca^{2+} , precedents for which are seen in

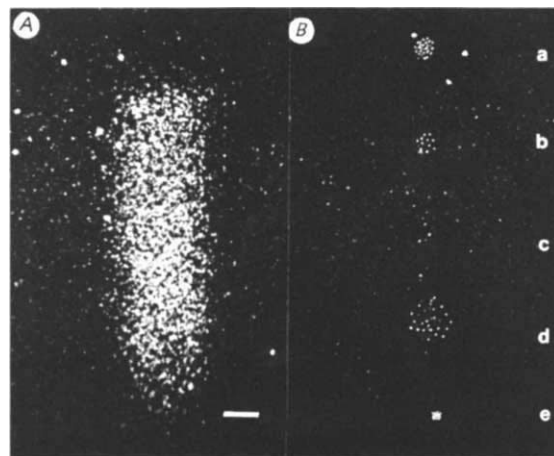


Fig. 4 Evaluation of image intensifier detection limits for small zones of sub-micromolar free calcium. **Frame A**, 10-s photograph of output phosphor of an EMI 9912 4-stage image intensifier viewing, through a microscope, an amoeba-sized (40 nl) cylindrical cuvette containing 5×10^{-7} M $[\text{Ca}^{2+}]_{\text{free}}$ buffer (see Fig. 2) injected to 5% of its volume with aequorin. Each punctate image represents one photoelectron. Scale bar, 100 μm . **Frame B**, 10-s exposure of background with five simulated images, a-e, superimposed. The photoelectron counts in these simulations were calculated from Fig. 2 using an estimate of 80 photoelectrons in a zone 91 μm diameter in frame A, an area which corresponds to the image of a spherical zone occupying 1% cell volume. The simulated images are: a, 1% at 5×10^{-7} M $[\text{Ca}^{2+}]$; b, 1% at 4×10^{-7} M; c, 1% at 2×10^{-7} M (not detectable above background); d, 10% at 2×10^{-7} M; e, 0.05% at 1×10^{-6} M. The detection limit for a spherical zone of 2×10^{-7} M $[\text{Ca}^{2+}]$ is between 1% and 10% cell volume. Higher $[\text{Ca}^{2+}]$ levels are detectable in zones occupying 1% of cell volume, assuming the zone is compact and quasi-spherical. Observations of amoebae under identical conditions failed to detect any signal¹⁰. It is therefore unlikely that persistent zones of free calcium above this detection limit occur *in vivo* in *Chaos*.

the modulation of the Ca^{2+} -sensitivity of actin-activated myosin ATP-ase by light chains³⁴ and in the cyclic nucleotide dependent regulation of Ca^{2+} -sensitivity of cardiac muscle³⁵. In this context it is interesting to note Taylor's observation¹⁴ that endoplasm in the rear of the cell responds more slowly to injections of threshold Ca^{2+} than anterior endoplasm. Alternatively it is possibly that the sensitivity to Ca^{2+} arises from a secondary mechanism which is not activated in the normal cell, except perhaps in the immediately sub-plasmalemmal layer^{9,31,33}, and that streaming is under the control of Ca^{2+} -independent processes such as myosin light chain phosphorylation^{36,37} myosin heavy chain phosphorylation by cofactor protein³⁸ and viscosity and gelation changes induced by 5'AMP^{6,39}.

Before the role of Ca^{2+} in motility control can be resolved it will be necessary to improve upon the measurements in *Chaos* and to extend the technique to other motile cells for which calcium control has been proposed. The demonstration of a calcium threshold with EGTA buffers can no longer be regarded as adequate evidence for control by modulation of the cytoplasmic concentration of free calcium.

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A monoclonal antibody for large-scale purification of human leukocyte interferon

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A clone of hybrid myelomas (NK2), secreting a mouse monoclonal antibody to human leukocyte interferon, has been isolated. The antibody neutralizes the antiviral activity of the interferon and, when covalently attached to a solid support and used as an immunoadsorbent, allows interferon purification of up to 5,000-fold in a single step.

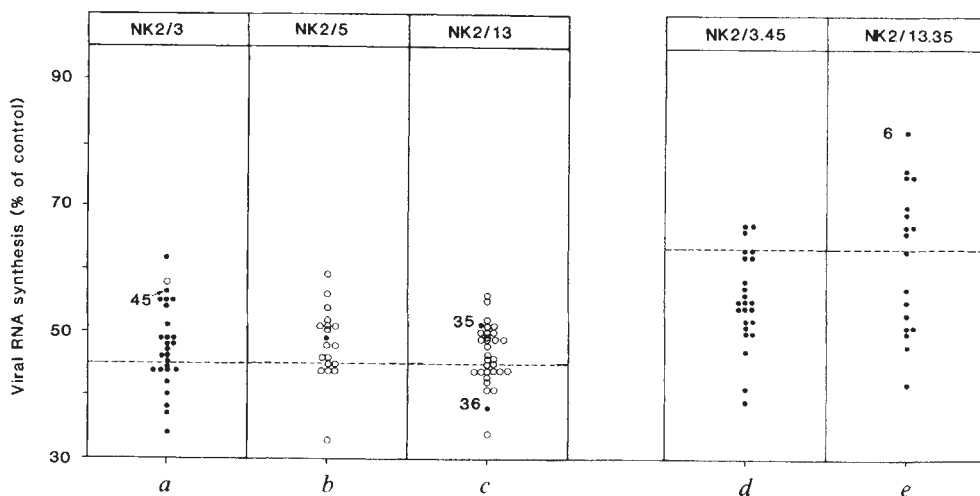
MONOCLONAL antibodies have been made by the spleen cell fusion technique of Köhler and Milstein¹ against a variety of antigens (reviewed in ref. 2). A theoretical advantage of the method is that it allows the production of specific antibodies to unpurified antigens. There is, however, no example of a monoclonal antibody that has been deliberately made to a predefined soluble antigen present at less than 1% of the immunogen. By using suitable screening methods it is possible to isolate rare hybrids producing antibody of the desired specificity, but this depends on screening with pure antigens. We describe here the properties of a monoclonal antibody to an antigen not available in pure form for screening assays and present in the immunizing material at a concentration of 0.1–1% of the total protein injected. This antigen is human leukocyte interferon, a protein (or group of proteins) that confers antiviral protection on human cells *in vitro* and *in vivo*.

There is currently considerable interest in the use of human leukocyte interferon as an antitumour and antiviral agent^{3,4}. Preparation of interferon for clinical use has required the development of large-scale production facilities^{5–7} coupled with extensive purification before administration to patients. Purification to homogeneity has recently been reported for two species of human interferon: fibroblast⁸ (2×10^8 U per mg) and leukocyte [obtained either from leukocytes⁹ ($2–4 \times 10^8$ U per mg) or from lymphoblastoid cells¹⁰ (2.5×10^8 U per mg)] and considerable progress has been made in the characterization of human (and mouse) interferons including amino acid compositions and amino-terminal sequences^{11–13}. However, the small scale of production prevents cheap

interferon from being readily available for characterization and clinical trials. Several groups have introduced immunoadsorbent column chromatography as one step in the purification of human interferon^{7,14–17}, but there are several problems connected with the production of antibodies against interferon. We considered that a homogeneous, monoclonal antibody would be advantageous in purifying interferon by immunoadsorption. In addition, interest in the interaction between interferon and the immune system^{18,19} has led to the need to assay low titres of leukocyte interferon in serum and other body fluids. For such purposes a radioimmunoassay, based on a monoclonal antibody, would be important. We report here the isolation of a hybrid myeloma, secreting antibody to human leukocyte interferon, and show that this antibody can be used for the purification of interferon by immunoadsorption.

The assays that have been used successfully in the production of monoclonal antibodies have been fast, reproducible, sensitive and simple. The lysis of antigen-coated red blood cells²⁰, binding on coated replica sheets²¹, radioactive binding assays²², haemagglutination assays (C. Milstein, personal communication) and radioimmunoassays²³, all fulfill these requirements and have been used in the isolation of monoclonal antibodies to haptens²⁰, cell-surface components²⁴, neuropeptides²³ and other antigens (see ref. 2). For interferon such assays would require large amounts of pure material or a radioimmunoassay (which is not available). Therefore, we were forced to use alternative assays based on a biological assay that is inherently complex and slow. Of the

Fig. 1 Properties of NK2 clones. Supernatants were assayed for anti-interferon activity and for the presence of secreted immunoglobulin. Hybrid cell culture supernatants were mixed with an equal volume of Dulbecco's modified Eagle's medium and 20% fetal calf serum (Gibco-Biotech) which contained 2-4 reference research units of interferon. After incubation at 37°C for 1 h, the fluids were assayed for residual interferon by the INAS₅₀ assay^{25,26}. Anti-interferon activity is shown by an increase in the level of viral RNA synthesis above that due to interferon alone (-----). Secreted immunoglobulin was detected by a reverse plaque assay²⁸. Open circles indicate that the supernatants were negative for secreted immunoglobulin, closed circles that they were positive (a, b, c) or untested (d, e).



available methods we chose to use the inhibition of nucleic acid synthesis (INAS₅₀) assay (refs 25, 26 and J. Morser, A. Meager, D.C.B. and D.S.S., manuscript in preparation).

Production and isolation of hybrid cells making monoclonal anti-interferon

Human interferon has been shown to be immunogenic in several heterologous species, but comparative studies¹⁴ have suggested that it is a much weaker immunogen in smaller animals (guinea pigs and rabbits) than in the large domestic animals (cows and sheep) usually used as a source of anti-interferon.

In the present study groups of mice and rats were immunized at 2-weekly intervals with lymphoblastoid interferon emulsified in complete Freund's adjuvant. A mouse with a titre (the dilution of serum that neutralized 50% of 20 U interferon) of log₁₀ 3.7 was selected for the fusion. Details of the immunization and fusion, as well as the results of a previous unsuccessful fusion, will be published elsewhere (J. Morser, A. Meager, D.C.B. and D.S.S., manuscript in preparation).

Three cultures (3, 5, 13), among several showing possible anti-interferon activity, were recloned in soft agar as described²⁷ and 48 clones of each were picked and grown in 2-ml cultures. Culture supernatants were tested for the presence of secreted mouse immunoglobulin using a reverse plaque assay²⁸ (spot-test) and for anti-interferon activity in the INAS₅₀ assay. The results shown in Fig. 1, as well as the results of many other assays not shown, clearly demonstrated that the level of neutralization of interferon by culture supernatants was just at (and sometimes beyond) the limits of sensitivity of the assay. At the critical stage of selection of a positive clone of NK2/13, the reverse plaque assay was essential.

To confirm that the anti-interferon activity was in fact due to immunoglobulin, and to determine the immunoglobulin class, ¹⁴C-lysine was incorporated into proteins secreted by selected clones and the extracellular medium analysed by SDS-polyacrylamide gel electrophoresis (PAGE) in reducing conditions²⁷. Only two major bands were visible in each case; one had the mobility of immunoglobulin heavy chain (γ, NK2/13.35; μ, NK2/13.36), the other co-migrated with the immunoglobulin light chain (NSI) that was run as a marker.

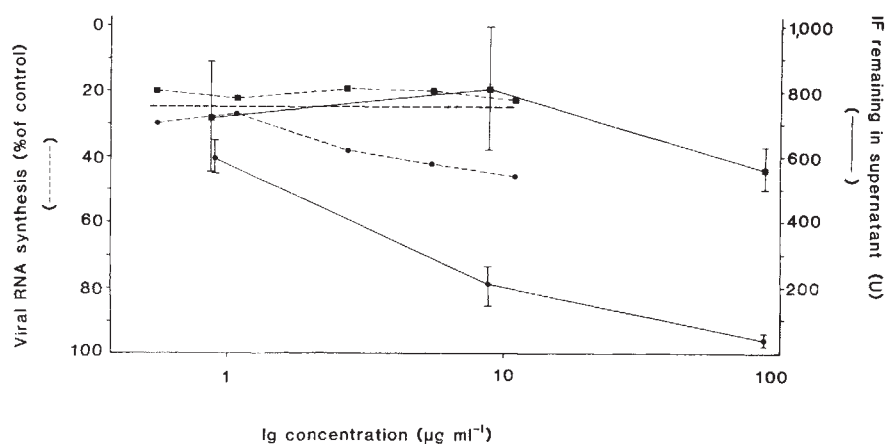


Fig. 2 Titration of anti-interferon activity of NK2 IgG. Anti-interferon activity was detected either by the standard neutralization assay or by the use of the indirect immunoprecipitation modification. Dilutions of IgG in phosphate-buffered saline (PBS) (10 μl) were incubated with about 720 U of lymphoblastoid interferon (10 μl) at 37°C for 5 h. Antibody-antigen complexes were then precipitated by the addition of carrier mouse IgG (5 μl normal mouse serum) and 75 μl of sheep anti-mouse IgG antiserum (slight antibody excess). After incubation at 37°C for 30 min and 4°C for 16 h the samples were centrifuged at 8,000g for 5 min to remove the antibody-antigen precipitate and the supernatant was assayed for its residual interferon content by the INAS₅₀ method. IIP assays were carried out in duplicate (NK2) or triplicate (control supernatant containing irrelevant monoclonal antibody) and the mean ± 1 s.d. is indicated. ●—●, NK2, neutralization; —●—●, control, neutralization; ●—●, NK2, IIP; —●—●, control, IIP.

Clone NK2/13.35 was recloned and the most strongly positive subclone (NK2/13.35.6) in the neutralization assay (Fig. 1e) was grown to mass culture and frozen in liquid nitrogen for long-term storage. This clone was also grown in the presence of ^{14}C -lysine and the radioactive secreted proteins analysed by SDS-PAGE. Two major bands were visible; one had the mobility of immunoglobulin γ -chain, the other had a mobility slightly lower than that of the NSI light chain. No band corresponding to the NSI light chain was detectable, indicating that the clone NK2/13.35.6 produces only the antibody-specific heavy and light chains (that is, NK2/HL in the nomenclature of ref. 29).

Precipitation of immune complexes improves the anti-interferon assay

The NK2/13.35.6 culture supernatant, although usually positive, was at best only sufficient to reduce the interferon activity to 25% of its control value using the interferon neutralization assay described in Fig. 1 legend. Some modifications to the assay were therefore tested. Concentration of culture supernatants 10–20-fold by ultrafiltration (Amicon XM 50) or by ammonium sulphate precipitation increased reproducibility, but the difference between a positive and a negative supernatant was not always convincing (data not shown). However, the results did show that the anti-interferon activity was associated with a high molecular weight (MW) component which was precipitated by 50% saturated ammonium sulphate. We also looked for, but could not detect, a synergistic effect on mixing supernatants from different cultures. Such an effect might result from the mixing of two non-neutralizing monoclonal antibodies, neither of which alone would be detectable. A similar phenomenon has been observed in studies of monoclonal antibodies to cell-surface antigens³⁰. A more successful modification to the assay was the introduction of an indirect immunoprecipitation (IIP) step (see Fig. 2 legend) in which interferon-anti-interferon complexes are removed (by the addition of carrier mouse immunoglobulin and anti-mouse immunoglobulin antiserum) before adding the interferon to the cells.

We also decided to use a purified IgG fraction prepared from the serum of mice bearing NK2 tumours to allow a higher concentration of immunoglobulin to be tested in the assay. (As the only clone from the NK2 fusion to be grown in mice was NK2/13.35.6 it will be abbreviated to NK2.) The

Table 1 Purification of crude interferon* by NK2 Sepharose immunoabsorbent chromatography

	Before	After
Volume (ml)	100	0.5
IF titre† (U ml ⁻¹)	7.2×10^4	1.4×10^7
Total U	7.2×10^6	7.0×10^6
A_{280}	2.2	0.09
Total protein‡ (mg)	220	0.04
Specific activity (U per mg)	3.3×10^4	1.8×10^8
Purification factor	1	5,300
Yield (%)	100	97

*Crude interferon was culture medium from stimulated Namalwa cells after removal of material that precipitated at pH 2.

†Error in IF assay estimated to be $\pm 30\%$.

‡Estimated assuming $A_{1\text{cm},280}^{1\%} = 10$. Most proteins have extinction coefficients in the range 5–15.

separation of the antibody-antigen complex formation from the neutralization part of the assay allowed the use of much higher levels of interferon as the interferon was diluted for assay after removal of the antibody-antigen complex. This greatly increased the precision of the assay because, by varying the dilution of the sample for interferon assay, we could always work near the midpoint of the dose response curve. This is impossible in the neutralization assay.

Using the IIP assay we found that at an antibody concentration of about $90 \mu\text{g ml}^{-1}$ it was possible to remove over 90% [696/720 U (reference research units as defined relative to the MRC reference standard preparation 69/19)] of input interferon. Plotting of the data from a neutralization experiment in the same figure allows a direct comparison of the two assays (Fig. 2), but note that the 45% of viral RNA synthesis obtained at $9 \mu\text{g ml}^{-1}$ NK2 IgG corresponds to the neutralization of about 80% of the interferon present (data not shown).

Towards a single-step purification of lymphoblastoid interferon

Anti-interferon antibodies have shown to be useful in the characterization of different interferons and the purification of both mouse and human interferons. Their use, however, has been limited by the fact that until recently only impure interferon preparations (such as that used in this study) were

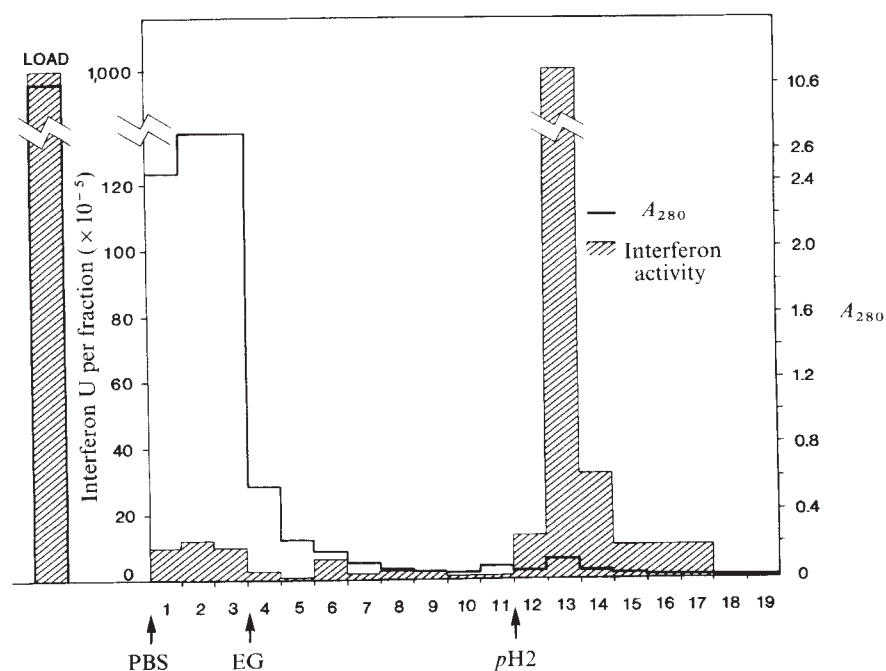


Fig. 3 Affinity chromatography of partially purified lymphoblastoid interferon. Lymphoblastoid interferon (10^8 U, 0.5 ml PBS, 1.9×10^7 U per mg) was loaded onto a 0.5 ml NK2-Sepharose column. Fractions of 0.5 ml were collected. The column was washed successively with PBS; 9 M ethane diol, 0.34 M NaCl and 0.0075 M Na phosphate buffer pH 7.4 (EG); 9 M ethane diol 0.3 M NaCl and 0.1 M citric acid (pH 2) as indicated. Fractions were assayed for interferon content and for protein content by measuring A_{280} and assuming an $A_{1\text{cm},280}^{1\%}$ of 10 (see Table 1 legend).

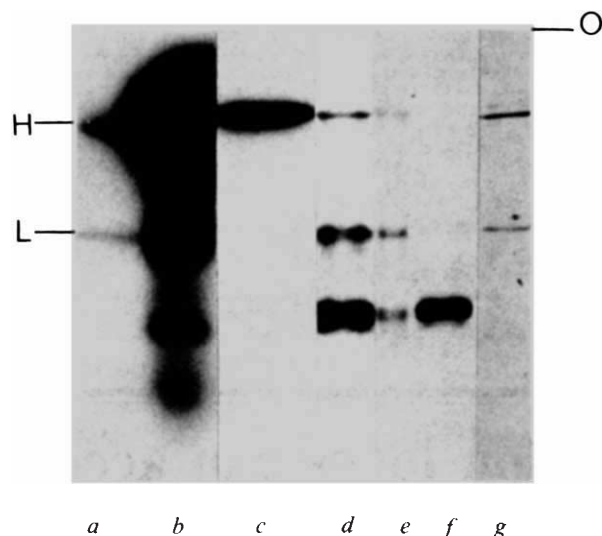


Fig. 4 Analysis of interferon samples described in the text. Interferon samples were iodinated using chloramine-T (ref. 33) (b, c) or the Bolton and Hunter reagent³⁴ (d, e, f). Aliquots were subjected to SDS-PAGE in reducing conditions³⁵ (14% acrylamide). The origin of migration in the separating gel is indicated (O). The mobilities of iodinated proteins (b-f) were compared with those of ¹⁴C-labelled immunoglobulin heavy (H) and light (L) chains (MOPC 21 IgG; a, g), and unlabelled bovine serum albumin. After electrophoresis the gels were dried and autoradiographed at -70 °C for various times (2 h-4 days). The figure is a composite of five autoradiographs (a-b, c, d, e-f, g) of two gels (a-c, d-g) run in identical conditions (viz. mobilities of the marker proteins in tracks a, g). The iodinated samples were as follows: c, partially purified interferon used for immunization. The only band visible co-migrated with serum albumin; b, gross over-exposure of c to show minor bands at about 21,000, 18,000 and 16,000 MW; d, IF-A, that is, interferon iodinated after one passage through NK2-Sepharose column; e, ¹²⁵I-IF-A not retained on second passage through NK2-Sepharose; f, ¹²⁵I-IF-A retained on NK2-Sepharose and eluted at pH 2.

available for immunization of animals, and the antisera thus obtained were directed mainly against contaminants in the interferon preparations. Now that leukocyte and lymphoblastoid interferons have been purified to homogeneity^{9,10}, the purified interferon could be used as the immunogen to obtain antisera of higher specificity. However, the existing methods have not allowed the purification of sufficient interferon for immunization of domestic animals required for large-scale production of conventional antiserum. The monoclonal antibody technique should allow us to bypass these problems, using low amounts of impure interferon to immunize small laboratory animals and, having selected a clone, to produce unlimited amounts of antibody of extremely high specificity and (presumably) high binding capacity. To prepare sufficient IgG for an immunoadsorbent column, NK2 cells were injected subcutaneously into BALB/c mice (10⁷ cells per mouse) and when tumours had developed, serum samples were collected and pooled. IgG was purified from a pool of 18 ml of serum by ammonium sulphate precipitation and DEAE-ion exchange chromatography as previously described for other monoclonal mouse IgG³¹. Purified NK2 IgG (14 mg) was coupled to 1 ml of CNBr-activated Sepharose (Pharmacia) and tested as an immunoadsorbent. The results, shown in Fig. 3, demonstrate that in a single passage through a 0.5-l column of NK2-Sepharose, a partially purified preparation of interferon (1.6 × 10⁶ U per mg) was purified almost 100-fold further. (The activity and specific activity of the interferon used was as stated by the suppliers and was not independently measured.) Insufficient material was available for accurate

determinations of protein concentrations, and so, as an alternative approach to assessing the purity of the interferon after a single passage through the NK2-Sepharose column (IF-A'), we radiolabelled an aliquot with ¹²⁵I and analysed it by SDS-PAGE (Fig. 4d). The autoradiograph of the gel showed a major band of MW about 18,000, with minor bands in the region of immunoglobulin heavy (~50,000) and light (~22,000) chains. The mobilities of the minor bands did not correspond exactly to those of the heavy and light chains of NK2 IgG and do not seem to be the result of NK2 IgG becoming detached from the Sepharose. A more likely possibility is that they are derived from the IgG produced by the lymphoblastoid (Namalwa) cells which might bind to the NK2 IgG on the column by nonspecific aggregation through the Fc. To show that the material retained on the column (IF-A) had indeed bound specifically to the NK2-Sepharose, we repurified ¹²⁵I-IF-A by a second passage through the same column. This time, the 18,000-MW band was specifically retained and eluted from the column, suggesting that at this stage the material was pure (Fig. 4f). An apparent MW of 17,500 or 18,000 for a leukocyte interferon component has been reported elsewhere^{7,9,17}.

As a more stringent test of the ability of the NK2-Sepharose to purify lymphoblastoid interferon, a crude sample of extracellular medium from stimulated Namalwa cells was applied to a 0.5-ml column. The results are shown in Table 1 and indicate a purification of about 5,000-fold in a single step. We have not investigated the purity of this material by SDS-PAGE but the specific activity is roughly the same as that of IF-A (1.2 × 10⁸ U per mg).

Future prospects

The results described above clearly demonstrate that the monoclonal antibody produced by NK2 is a very useful reagent for human lymphoblastoid interferon purification. Because the antibody is the product of a transplantable tumour and can easily be purified from the serum of tumour-bearing mice, there is no limit to the amount of NK2 antibody that could be made without any further need for interferon as immunogen. Thus, the protocols described here could easily be scaled up for the large-scale purification of interferon. An important advantage of the monoclonal antibody is that this method of purification should be equally applicable to interferon from human leukocytes or indeed from any other source (such as *Escherichia coli*³²) producing human interferon, provided it contains the NK2 antigenic determinant.

The recovery of interferon activity following iodination was quantitative and the ¹²⁵I-labelled interferon retained its biological activity after purification on NK2-Sepharose. Thus, it may be possible to use this material, in conjunction with the NK2 IgG, to develop a radioimmunoassay for interferon. Such an assay would greatly simplify the type of experiments described here as well as the purification of interferon and the analysis of interferon in biological fluids and in products of animal or bacterial cell cultures.

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Deletions in the constant region locus can account for switches in immunoglobulin heavy chain expression

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Deletions spanning portions of the heavy chain constant region (C_H) locus in murine plasmacytomas are revealed by hybridization with cloned immunoglobulin DNA molecules. The pattern of deletions suggests that a lymphocyte clone switches expression of C_H genes by successive deletions and that the probable C_H gene order is 5' μ - γ 3- γ 1- γ 2b- γ 2a- α 3'.

THE immunoglobulin genes (reviewed in refs 1, 2) represent a unique system in which single polypeptide chains are encoded by more than one gene. An immunoglobulin molecule contains either κ or λ light chains and, in mice, one of eight types of heavy chain (μ , δ , α , γ 1, γ 2a, γ 2b, γ 3, ϵ), the heavy chain defining the immunoglobulin class (IgM, IgD, IgA, IgG1, IgG2a, IgG2b, IgG3, IgE). Chains of each type are composed of a constant (C) region and a region of variable sequence (V region) which mediates antigen binding. V and C regions are encoded separately in the germ line^{3,4}, and a functional immunoglobulin gene is generated within a lymphocyte clone by a recombination event⁵ which associates a particular V and C gene⁶. This somatic rearrangement step, which is well documented for light chain genes^{7–10}, has recently been described for α and μ genes^{11–13}. Rearrangement within the heavy chain locus is presumably more complex than that within the κ or λ loci, because the multiple V_H genes are shared by the different C_H genes¹⁴, which occur in an unknown order within a tight cluster^{15,16}.

A fundamental puzzle posed by the immune system is that a lymphocyte clone can switch from expression of one class of immunoglobulin to another (for example, IgM to IgG1) while maintaining its V-region specificity^{17–25}. This suggests that a single V_H region can associate sequentially with different C_H regions within the one cell lineage. Switching of C_H regions may be restricted to a single chromosome, because only one allele of a heavy chain gene is expressed in a given cell ('allelic exclusion')^{26,27} and μ and δ chains in the same cell are derived from one chromosome²⁸. Switching can be studied by comparing the DNA of different murine plasmacytomas, each of which synthesizes an immunoglobulin of a single class.

Several plausible models for heavy chain expression have been put forward (see refs 29, 30 for reviews). In one^{31,32}, variable splicing of a putative precursor RNA spanning the entire C_H locus generates different heavy chain mRNAs. This

model now seems untenable as a general case, because the precursor RNAs seem to bear single C_H sequences³³. In a second model^{21,34}, copies of a particular V_H region are inserted near each C_H gene. Evidence against this model is that two plasmacytomas have been shown not to have an increased copy number for the V_H gene they express¹³. In a third model³⁵, one V_H gene undergoes a series of shifts to associate it with different C_H genes. Finally, in the deletion model^{36,37} (Fig. 1), the DNA between a particular V_H gene and the first C_H gene to be expressed is excised and subsequent C_H switching occurs by successive deletion of C_H genes and the intervening DNA. Deletions are not expected on any other model.

Recent evidence⁹ implicates a deletion mechanism for association of the single V_λ and C_λ genes. Honjo and Kataoka³⁷ proposed earlier that C_H switching occurs by deletions confined to one of the two homologous chromosomes. Their kinetic hybridization (C_0t) analysis with uncloned cDNA probes suggested that the concentration of certain C_γ sequences in DNA of particular plasmacytomas was half that in DNA of non-lymphoid tissues. Their results were consistent with deletions operating on C_H genes arranged in the order (μ , γ 3) γ 1- γ 2b- γ 2a- α . Twofold differences are, however, just resolvable by kinetic hybridization and such results might be affected by probe impurities or cross-hybridization between different C_γ sequences.

We have recently used cloned μ and α cDNA probes³⁸ to establish that deletions are associated with rearrangement of heavy chain genes¹³. Several V_H genes were absent from one plasmacytoma, and C_μ sequences were either partially or totally deleted from seven IgG- or IgA-secreting plasmacytomas each of which retained the C_α gene. Those results stimulated us to examine different C_γ genes in the same tumours. We report here that C_γ genes undergo somatic rearrangement and that particular C_γ genes and flanking

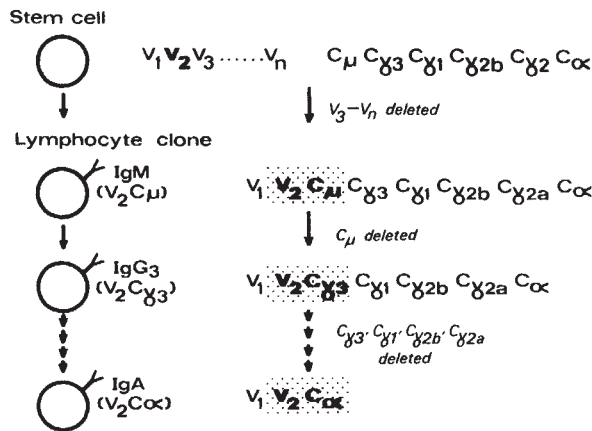


Fig. 1 Deletion model for somatic rearrangement of immunoglobulin heavy chain genes. The germ-line arrangement of V_H and C_H genes in a stem cell is compared with the corresponding arrangements postulated for a lymphocyte clone which synthesizes a μ heavy chain and then switches successively to synthesis of γ 3 and α chains, each type of heavy chain bearing the same V_H sequence (V₂). The functional V_HC_H gene is shown bold. C_H genes are shown in the order favoured by the data presented in the text; the location of C₃ and C₂ is unknown. Embryo serves as a model of the germ line in the present study and various plasmacytomas represent lymphocytes at different stages of the switching process.

sequences have been deleted in certain plasmacytomas. The probable C_H gene order can be inferred from the deletions. The pattern of deletions is complex and cannot be accounted for by events confined to a single chromosome. Deletions extend close to rearranged, presumably active C_γ genes. The results strongly support a deletion model for switches in heavy chain expression. Immunoglobulin gene expression thus seems to be regulated by an unprecedented mechanism involving sequential excision of portions of the genetic material.

Each C_γ gene can be detected on a separate restriction fragment

We first established that we could score the four C_γ genes on restriction endonuclease fragments of mouse DNA. The probes were cloned cDNA molecules bearing C_{γ1}, C_{γ3} and C_{γ2a} sequences³⁸, and for C_{γ1} and C_{γ3}, cloned gene fragments (see below). We expected that the C_{γ2a} probe would detect the C_{γ2b} as well as C_{γ2a} gene, because the two C_{γ2} amino acid sequences exhibit homology^{39,40} and their nucleotide sequences cross-hybridize⁴¹. We fractionated *Eco*RI endonuclease digests of embryo DNA by gel electrophoresis, blotted the fragments onto nitrocellulose filters⁴² and revealed specific fragments by hybridization with ³²P-labelled probes. The C_{γ1} and C_{γ2b} genes lie in separate *Eco*RI fragments nearly 7 kilobase pairs long, neither of which contains another C_γ gene⁴³⁻⁴⁶. Figure 2A shows that, as expected, the C_{γ1} probe hybridized strongly to a ~7-kilobase fragment, as did the C_{γ2a} probe, presumably by cross-hybridization with the C_{γ2b} gene. The C_{γ2a} probe also strongly labelled a ~23-kilobase fragment and weakly labelled a ~19-kilobase fragment, whereas the C_{γ3} probe gave the opposite result. We conclude that the C_{γ2a} gene lies in the ~23-kilobase fragment and the C_{γ3} in the ~19-kilobase fragment. Partial cross-hybridization of C_{γ2a} and C_{γ3} sequences would be expected⁴¹. C_{γ2} and C_{γ1} sequences do not cross-hybridize significantly under our conditions, because the C_{γ1} probe did not label the ~23-kilobase C_{γ2a} fragment. To confirm this, we hybridized each probe to *Hind*III fragments of embryo DNA. The results (Fig. 2B) indicate that the C_{γ2a} probe barely hybridized to the fragment bearing the C_{γ1} gene, and that the C_{γ1} probe did not hybridize to fragments bearing the C_{γ2a} or C_{γ2b} genes. The pattern of cross-hybridization observed is in accord with results⁴¹ from solution hybridization, and both the cross-hybridizations and the fragment sizes detected are entirely consistent with results from cloned C_γ sequences (refs 43-46 and J.M.A., E. Webb, S. Gerondakis and S.C., manuscript in preparation).

C_γ genes undergo somatic rearrangement

Because rearrangement of immunoglobulin genes seems to be a prerequisite for expression, we sought evidence of changes in context for C_γ genes by comparing restriction fragments bearing C_γ genes in plasmacytoma DNA with those in embryo. Figure 2C shows that C_{γ3} sequences in the IgG3 producer Y5606 (track b) occur in an *Eco*RI fragment not present in embryo (track a). This suggests that sequences surrounding the C_{γ3} gene have altered during development. Similarly, *Hind*III digests (Fig. 2D) revealed that C_{γ1} sequences in the IgG1 producer MOPC 21 (track c) occur in two fragments, each different in size from that in embryo (track a). The C_{γ1} gene is not cut by *Hind*III (track a and our unpublished results). Thus, there seem to be two types of rearranged C_{γ1} sequences in MOPC 21, as found previously for C_μ sequences in an IgM producer and for C_α in two IgA producers¹³. What the two C_{γ1} sequences represent is unclear; they apparently do not correspond to different alleles because our data suggest that MOPC 21 cells contain only one copy of the C_{γ1} gene (see below). Cellular heterogeneity could explain the two bands, if the sequence flanking the C_{γ1} gene underwent an additional alteration after the cloning of the

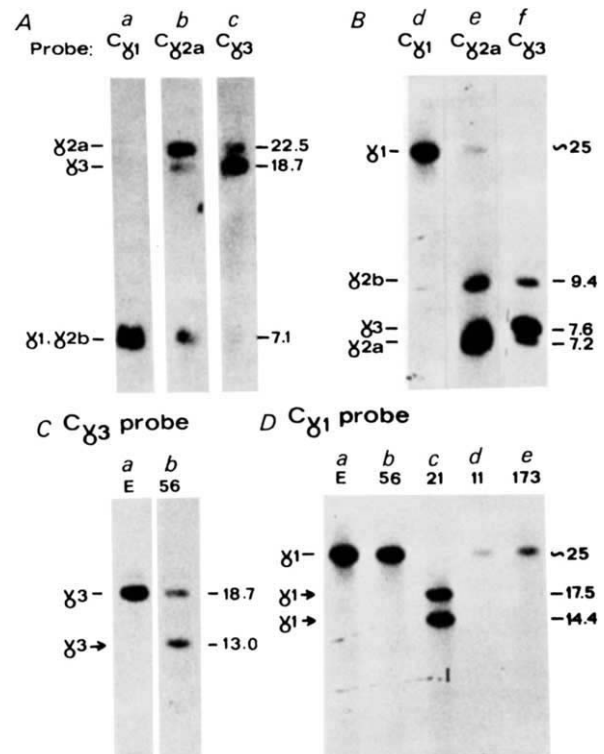


Fig. 2 Identification of restriction fragments bearing C_γ genes in germ-line context and after rearrangement. Restriction fragments (15 μg) of DNA from BALB/c embryos and plasmacytomas were fractionated by electrophoresis on 0.7% agarose gels, blotted on to nitrocellulose filters⁴² and then hybridized with ³²P-labelled nick-translated³⁷ probes as described previously¹³. Autoradiography was carried out at -70 °C with an intensifying screen³⁸. **A**, *Eco*RI fragments of embryo DNA hybridized with cloned C_{γ1}, C_{γ2a} and C_{γ3} cDNA probes as indicated. **B**, *Hind*III fragments of embryo DNA hybridized to a genomic C_{γ1} probe (track d) (fragment f in Fig. 4B), the C_{γ2a} cDNA probe (track e) and the C_{γ3} cDNA probe (track f). The C_{γ3} and C_{γ2a} fragments clearly resolved on a shorter exposure. **C**, C_{γ3}-bearing *Eco*RI fragments of DNA from embryo (track a) and the IgG3 producer Y5606 (track b) detected with a genomic C_{γ3} probe (fragment b in Fig. 4A). The same pattern was obtained with the C_{γ3} cDNA probe. **D**, C_{γ1}-bearing *Hind*III fragments of DNA from embryos (track a) and the IgG3 producer Y5606 (track b), the IgG1 producer MOPC 21 (track c) the IgG2b producer MPC 11 (track d), and the IgG2a producer MOPC 173 (track e), detected with a genomic C_{γ1} probe (fragment f in Fig. 4B). In C and D, fragments bearing rearranged C_{γ3} and C_{γ1} sequences are indicated by arrows. Fragment sizes are given in kilobases. The cloned cDNA sequences have been described in detail elsewhere³⁸. The bottom of autoradiograms in this and subsequent figures are not shown because no smaller fragments were detected.

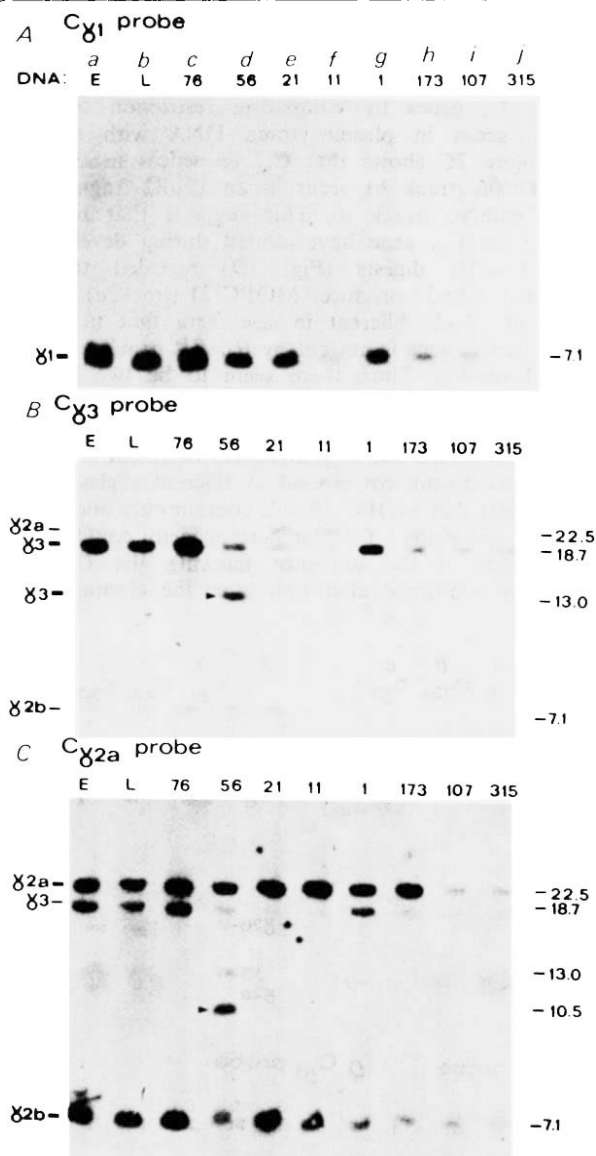


Fig. 3 Levels of C_γ sequences in embryo, liver and different plasmacytomas. The filters bore fractionated *Eco*RI digests of DNA (15 μ g) from BALB/c embryos (track a), adult liver (track b) and the IgM producer HPC 76 (track c), the IgG3 producer Y5606 (track d), the IgG1 producer MOPC 21 (track e), the IgG2b producer MPC 11 (track f), the IgG2a producers HOPC 1 (track g) and MOPC 173 (track h), and the IgA producers S107 (track i) and MOPC 315 (track j). The probes were: A, a genomic $C_{\gamma 1}$ probe (fragment d in Fig. 4B); B, a genomic $C_{\gamma 3}$ probe (fragment b in Fig. 4A); C, a $C_{\gamma 2a}$ cDNA probe. Cloned $C_{\gamma 1}$ and $C_{\gamma 3}$ cDNA probes gave the same results as the genomic probes. Fragments bearing C_γ genes are indicated and their sizes given in kilobases. Rearranged sequences are indicated by arrows. We have not determined whether the rearranged fragment detected by the $C_{\gamma 2a}$ probe in Y5606 DNA corresponds to a $C_{\gamma 2a}$ or a $C_{\gamma 2b}$ sequence. References to the origin of the plasmacytomas are given elsewhere¹³.

C_γ genes are deleted in IgA- and IgG-producing plasmacytomas

We examined DNA from eight plasmacytomas for deletions involving C_γ genes by scoring *Eco*RI fragments with different C_γ probes (Fig. 3). Scanning across Fig. 3 reveals that the level of each C_γ gene in various plasmacytomas differs strikingly. For example, Fig. 3A shows that whereas $C_{\gamma 1}$ sequences are present in the IgM producer HPC 76 (track c) at about the same level as in embryo (track a) or liver (track b), the two IgA producers S107 and MOPC 315 (tracks i and j) contain extremely low levels and the IgG producers (tracks d-h) contain intermediate to very low levels. We can discount degradation as an explanation because each DNA preparation was of high molecular weight (>80 kilobases), and each digest yielded strong, well defined bands for the ~23-kilobase $C_{\gamma 2a}$ fragment (Fig. 3C) and/or for the C_α gene¹³, as well as for V_H genes¹³. Moreover, deletions were also apparent in *Hind*III digests (Fig. 2D), as shown, for example, by the virtual absence of $C_{\gamma 1}$ sequences in the IgG2b producer MPC 11 (track d) and the IgG2a producer MOPC 173 (track e).

Comparing equivalent tracks in Fig. 3A, B and C allows assessment of the level of different C_γ genes in a particular tumour. It is clear that deletions in the IgA producers S107 (track i) and MOPC 315 (track j) involve all four C_γ genes. In contrast, only certain C_γ genes have been deleted from particular IgG producers. For example, the IgG1 producer MOPC 21 (track e) contains much lower levels of $C_{\gamma 3}$ sequences than of $C_{\gamma 1}$, $C_{\gamma 2a}$ or $C_{\gamma 2b}$.

We determined the copy number of each C_γ gene in the different plasmacytomas by densitometry of autoradiographs like those in Fig. 3. Because the same amount of DNA was loaded for each sample and hybridization was compared on the same filter, the autoradiographic signal is proportional to gene concentration^{13,47}. Dividing the signal by that given for embryo DNA gives a measure of the copies per haploid genome, assuming a single copy per haploid genome in embryo DNA. This assumption has been validated for the C_μ gene¹³ and probably also holds for each C_γ gene, because only one *Eco*RI or *Hind*III fragment of embryo DNA was detected for each C_γ gene. Table 1 presents values for the copies per haploid genome for each C_γ gene, for $C_{\gamma 1}$ and $C_{\gamma 3}$ flanking sequences described below, and for the C_α gene. We scored each C_H sequence in the different plasmacytomas at least twice and the quantitative results were reproducible. For example, scoring the $C_{\gamma 1}$ gene with a cDNA and a genomic probe gave nearly identical results (Table 1) and, in general, values from separate experiments agreed within 25%, except for very faint bands. We have shown elsewhere¹³ that the very low levels of residual sequences detected in some lines probably result from a few per cent contamination of the transplanted tumours by non-lymphoid cells.

Deletions span large sequences on the 5' side of C_γ genes and extend close to active genes

It is important to establish whether the deletions include sequences between C_H genes. To permit studies with sequences flanking C_γ genes, we searched a library of clones bearing large fragments of mouse embryo DNA⁴⁸ and have identified a clone (G1.1) bearing a $C_{\gamma 1}$ sequence and another (G3.1) bearing a $C_{\gamma 3}$ gene (J.M.A., E. Webb, S. Gerondakis and S.C., manuscript in preparation). Their identity rests on the extent of hybridization with authenticated³⁸ $C_{\gamma 1}$, $C_{\gamma 2a}$ and $C_{\gamma 3}$ cDNA probes, on the absence of restriction sites characteristic of $C_{\gamma 2a}$ cDNA³⁸ and the $C_{\gamma 2b}$ gene^{45,46}, on an excellent correlation of the restriction map of G1.1 with those of two other $C_{\gamma 1}$ -bearing clones^{43,44} and on a good match between restriction sites within the $C_{\gamma 3}$ gene of G3.1 and sites³⁸ within $\gamma 3$ cDNA. The diagrams in Fig. 4 show that G3.1 contains 8.6 kilobase of DNA on the 5' side of the $C_{\gamma 3}$ gene, with respect to its

MOPC 21 (P3) cell line. The observation that a rearranged $C_{\gamma 1}$ gene was found only in the IgG1 producer (Fig. 2D) and a rearranged $C_{\gamma 3}$ gene only in the IgG3 producer (see Fig. 3B) suggests that these changes are related to expression of those genes. We did not find evidence of a rearranged $C_{\gamma 2b}$ gene in the IgG2b producer MPC 11 or of a rearranged $C_{\gamma 2a}$ gene in the IgG2a producer MOPC 173, in either *Hind*III digests (not shown) or *Eco*RI digests (see Fig. 3C). We surmise that changes in those tumours occur beyond the nearest *Hind*III and *Eco*RI sites

Table 1 Quantification* of C_H genes and flanking sequences in DNA from different plasmacytomas

Plasmacytoma	5' to $C_{\gamma 3}$	$C_{\gamma 3}$ gene		5' to $C_{\gamma 1}$	$C_{\gamma 1}$ gene		$C_{\gamma 2b}$ gene	$C_{\gamma 2a}$ gene	C_{γ} gene
	probe a	cDNA	probe b	probe c	probe e	probe f	probe d	cDNA	cDNA
HPC 76 (IgM)	0.70	1.1	1.0	1.0	1.4	1.4	1.3	1.2	1.2 ± 0.2
Y5606 (IgG3)	0.04	0.12	0.12	0.48	0.51	0.41	0.42	0.20	0.9 ± 0.3
		0.14 [†]	0.17 [‡]					0.16 [¶]	
MOPC 21 (IgG1)	<0.01 [†]	0.05	0.04	0.06	0.45	0.31	0.38	1.1	1.3
				0.05 [§]					1.6 ± 0.1
				0.01 [¶]					
MPC 11 (IgG2b)	0.02	0.03	0.02	0.05	0.04	0.03	0.05	0.25	1.1
MOPC 173 (IgG2a)	0.04	0.08	0.09	0.12	0.09	0.07	0.10	0.07	0.74
HOPC 1 (IgG2a)	0.15	0.31	0.39	0.42	0.39	0.32	0.32	0.11	0.52
S107 (IgA)	0.03	0.05	0.05	0.09	0.09	0.06	0.08	0.07	0.08
MOPC 315 (IgA)	0.02	0.04	0.06	0.08	0.06	0.05	0.04	0.04	0.06
									0.8 ± 0.2

DNA was isolated^{1,3} from plasmacytomas (fully referenced elsewhere^{1,3}) grown as subcutaneous tumours except where otherwise indicated. Probe a was the embryonic sequence extending 8.6 kilobases from the $C_{\gamma 3}$ gene (fragment a in Fig. 4A); the results are the average of three experiments. The cDNA probe for the $C_{\gamma 3}$ gene was a 0.53-kilobase *Hinf* fragment isolated by polyacrylamide gel electrophoresis from the cDNA insert of plasmid pY5606y3.15 (ref. 38). Probe b was the 4.8-kilobase *Bam*HI/*Hind*III fragment from G3.1 (fragment b in Fig. 4A). Probe c was the 10-kilobase *Hind*III/*Eco*RI fragment from G1.1 (fragment c in Fig. 4B); the results are the average of two experiments. Probe e was the 2.8-kilobase *Eco*RI/*Bgl*II fragment from G1.1 (fragment e in Fig. 4B) obtained from the subclone pG1.1R. Probe f was the 1.4-kilobase *Bgl*II/*Eco*RI* fragment from G1.1 (fragment f in Fig. 4B), obtained from the subclone pG1.1R. Probe d was the 5-kilobase *Eco*RI/*Eco*RI* fragment from G1.1 (fragment d in Fig. 4B), which had been subcloned into pBR322 (plasmid pG1.1R). The cDNA probes for the $C_{\gamma 2b}$ and $C_{\gamma 2a}$ genes were 0.42- and 0.29-kilobase $C_{\gamma 2a}$ fragments from the cDNA plasmid pM173y2a.15 (ref. 38); results are the average of two experiments. The cDNA probes for the C_{γ} gene were fragments from the cDNA plasmid pS107y4 (ref. 38); results are the average of six experiments, including determinations made previously¹³, and the standard deviation is given. Variation between experiments was greater than for C_{γ} fragments, perhaps due to the smaller size of the 3' C_{γ} -bearing *Eco*RI fragment (4.7 kilobases) (ref. 13). The $C_{\gamma 2b}$ gene was detected by cross-hybridization to $C_{\gamma 2a}$ probes (see text).

*Results are expressed as copies per haploid mouse genome, assuming one copy of each C_H gene in the germ line. Intensities of bands on autoradiographs were measured using a Canalco model J microdensitometer. For each probe, DNAs were compared on the same filter and the values from each related to that in embryo DNA. Where more than one fragment was detected in a particular DNA, the values are listed in order of decreasing fragment size. Values for rearranged sequences are italicized.

[†]DNA was isolated from MOPC 21 cells (P3 clone⁵⁶) grown in tissue culture. Equivalent experiments with DNA from the P3 line grown as subcutaneous tumours gave 0.022 ± 0.004 copies, which presumably indicates about 2% contamination of that preparation by DNA from non-lymphoid cells.

[‡]The 13.0-kilobase fragment in track d, Fig. 3B.

[§]The 7.4-kilobase fragment in track e, Fig. 4B. As this fragment contains about 5% of the label in embryo DNA, if it corresponds to one gene equivalent of the sequence in a tetraploid cell (see text), it bears $4 \times 5\% = 20\%$ of the sequences in one equivalent of the 10-kilobase fragment, or 2 kilobases of the sequences.

[¶]A 4.6-kilobase fragment in track e, Fig. 4B, visible on longer exposures.

||DNA was isolated from MOPC 21 cells (P3 clone⁵⁶) grown in tissue culture.

^{¶¶}The 10.5-kilobase fragment in track d, Fig. 3C, arbitrarily nominated as bearing $\gamma 2b$ rather than $\gamma 2a$ sequences.

transcription, whereas G1.1 contains 13.2 kilobases on the 5' side of the $C_{\gamma 1}$ gene. Neither cloned sequence contains another C_{γ} gene or the C_{μ} or C_{α} genes (our unpublished results).

Using fragments from the cloned embryonic sequences as probes, we scored plasmacytoma DNAs for specific regions flanking the $C_{\gamma 3}$ and $C_{\gamma 1}$ genes. One probe, fragment a in Fig. 4, extends 8.6 kilobases from the $C_{\gamma 3}$ gene on its 5' side. Figure 4A shows that the level of this sequence was comparable in embryo, liver and the IgM secretor HPC 76 (tracks a-c) but markedly reduced in all the other plasmacytomas, and the data in Table 1 confirm this. As the pattern of deletions was generally the same as that of the $C_{\gamma 3}$ gene (Fig. 3B), we conclude that deletions involving the $C_{\gamma 3}$ gene extend at least 8.6 kilobases on the 5' side of the gene. Significantly, the embryonic sequence was virtually absent from the IgG3 producer Y5606 (track d, Fig. 4A and Table 1). Hence, the deletion extends close to the $C_{\gamma 3}$ gene in Y5606. Other data (S.C. and J.M.A., manuscript in preparation) suggest that the deletion stops about 2 kilobases from the $C_{\gamma 3}$ gene, as indicated by the broken line at the top of Fig. 4A.

Certain deletions also involved embryonic sequences flanking the $C_{\gamma 1}$ gene. Figure 4B shows that the sequences lying between 3.2 and 13.2 kilobases on the 5' side of the $C_{\gamma 1}$ gene (fragment c) were deleted in several of the tumours and the deletions generally paralleled those of the $C_{\gamma 1}$ gene. Deletions involving the $C_{\gamma 1}$ gene must therefore extend to at least 13 kilobases on the 5' side of the gene. Significantly, very little of this sequence remained in MOPC 21 DNA, whereas the sequence lying between 0.4 and 3.2 kilobases on the 5' side of the $C_{\gamma 1}$ gene (fragment e in Fig. 4B) was present in MOPC 21 at a level comparable with that of the $C_{\gamma 1}$ gene (Table 1). Hence, as indicated by the broken line in Fig. 4B, the deletion in MOPC 21 must stop slightly to the left of the *Eco*RI site separating fragments c and e. If so, a new *Eco*RI fragment which hybridizes weakly with fragment c should have been generated in MOPC 21 DNA by the deletion. Such a fragment is indicated by an arrow in Fig. 4B (track e). From the extent of its labelling, we calculate that this fragment contains about

2 kilobases of the sequences in fragment c (see footnote §, Table 1). Hence, if this residual sequence lies adjacent to the *Eco*RI site, which is 3.2 kilobases from the $C_{\gamma 1}$ gene, the deletion must extend to within about $3.2 + 2.0 = 5.2$ kilobases of the gene.

Although the deletion model predicts excision of sequences on the 5' side of an active C_H gene (Fig. 1), other models^{21,35} postulate insertion of a V_H gene into that region. In multicopy insertion²¹, for example, a V_H gene would be inserted on the 5' side of each C_H gene in every plasmacytoma. Such insertions should alter the size of fragments revealed with a flanking region probe. Except for the weakly labelled MOPC 21 fragments in Fig. 4B, no fragments of altered size were detected in any plasmacytoma DNA with fragments a, c or e as probes. Hence, the results in Fig. 4 strongly support the deletion model but not the other models.

Deletions are frequently not confined to single alleles of C_{γ} genes

As plasmacytomas are near tetraploid⁴⁹, we can estimate the number of copies of each C_H gene per cell by multiplying the values in Table 1 by 4. Most plasmacytomas are actually hypotetraploid, however, and the chromosome number varies somewhat in different lines⁴⁹. The lines examined here seem to retain at least three and probably four copies of the C_H -bearing locus, because the level of C_{α} sequences in each line was at least 0.8 times that in embryo (Table 1). To compensate for any variation between lines in ploidy and therefore in the ratio of C_H -bearing to total chromosomes, it seems useful to relate the values for each C_H gene to that of C_{α} . Figure 5 thus displays the values from Table 1 for the copy numbers of C_{γ} genes, as well as values¹³ for the C_{μ} gene, normalized to 4.0 copies of the C_{α} gene per cell. We have estimated the probable level of sequences derived from non-lymphoid cells contaminating the transplanted tumours (ref. 13 and Fig. 5 legend) and the filled portions of the bars represent our estimates for the gene copy numbers actually in plasmacytoma

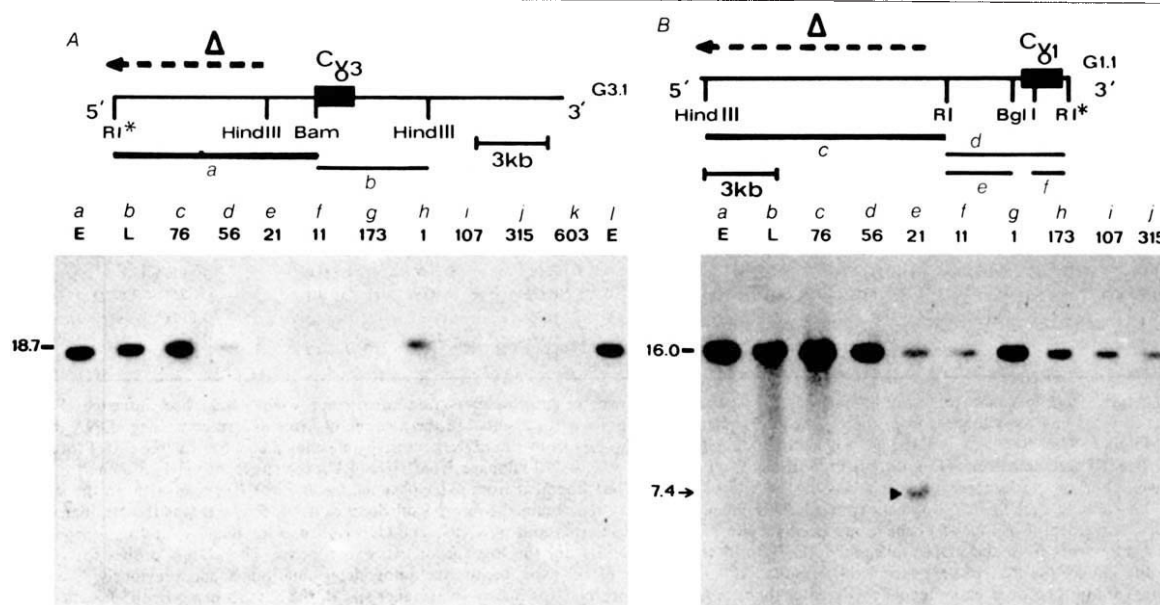


Fig. 4 Levels of embryonic sequences flanking $C_{\gamma 3}$ and $C_{\gamma 1}$ genes in embryo, liver and different plasmacytomas. Detailed maps of clones G3.1 and G1.1 and evidence on the position and orientation of the C_{γ} genes will be reported elsewhere. Only those restriction sites relevant to the isolation of fragments used in this study are indicated; 'RI' indicates an *EcoRI* site present in mouse DNA and 'RI*' an *EcoRI* site generated by the cloning technique⁴⁸. The broken lines indicate sequences deleted (Δ) in two plasmacytomas (see text). The orientation refers to the direction of transcription of the C_{γ} gene. **A**, Sequences detected with fragment *a* from G3.1. The filters contained fractionated *EcoRI* digests of DNA (15 μ g) from BALB/c embryos (track *a* and *l*), adult liver (track *b*) and plasmacytomas HPC 76 (track *c*), Y5606 (track *d*), MOPC 21 (track *e*), MPC 11 (track *f*), MOPC 173 (track *g*), HOPC 1 (track *h*), S107 (track *i*), MOPC 315 (track *j*) and the IgA producer McPC 603 (track *k*). **B**, Sequences detected with fragment *c* from G1.1. The filter contained *EcoRI* digests of DNA from embryo (track *a*), adult liver (track *b*) and plasmacytomas HPC 76 (track *c*), Y5606 (track *d*), MOPC 21 (track *e*), MPC 11 (track *f*), HOPC 1 (track *g*), MOPC 173 (track *h*), S107 (track *i*) and MOPC 315 (track *j*). A 16-kilobase (kb) fragment is detected because that is the distance to the next *EcoRI* site to the left of that shown in the cloned sequence. A fragment (7.4 kilobases) bearing rearranged sequences is indicated by an arrow; two other weakly labelled fragments of 11.3 and 4.6 kilobases were also visible on longer exposures.

DNA. The reproducibility of different experiments leads us to conclude that our results can distinguish between 0 and 1, 1 and 2, or 2 and 4 copies of a C_H gene but probably not between 3 and 4 copies.

If deletions occurred on only one chromosome in a diploid lymphocyte, the subsequent chromosome doubling in the plasmacytomas would leave either two or four copies per cell for each C_H gene. Figure 5 shows that this is usually not the case. For example, it seems clear that all copies of the $C_{\gamma 2b}$ gene must be deleted from MOPC 173, HOPC 1, S107 and MOPC 315. We conclude that deletions are frequently not confined to one allele of C_H genes. On the other hand, deletion of both alleles cannot be obligatory, as the IgG2a producer HOPC 1 retains substantial amounts of the C_{μ} , $C_{\gamma 3}$ and $C_{\gamma 1}$ genes and MPC 11 retains two copies of the C_{μ} gene. It seems significant that the C_{μ} genes remaining in both these lines have an alteration in the C_{μ} 5'-flanking sequence concerned with C_H switching (ref. 13 and our unpublished results).

Probable C_H gene order is μ - $\gamma 3$ - $\gamma 1$ - $\gamma 2b$ - $\gamma 2a$ - α

The deletions provide evidence on the C_H gene order, independent of any conclusions regarding switching. The rationale is that if two or more genes are deleted from the DNA of any plasmacytoma line, they are taken to be linked, the assumption being that this results from a single deletion rather than two independent ones. Thus, the virtual absence of C_{μ} and all C_{γ} sequences from the two IgA producers strongly suggests that the C_{α} gene lies to one side of the locus. Similarly, the absence of genes other than $C_{\gamma 2a}$ and C_{α} in the IgG2a producer MOPC 173 strongly suggests that $C_{\gamma 2a}$ and C_{α} are coupled, leading to the order (μ , $\gamma 3$, $\gamma 1$, $\gamma 2b$)- $\gamma 2a$ - α . The absence of $C_{\gamma 3}$ and $C_{\gamma 1}$ genes from MPC 11 DNA argues that they are coupled, and linkage of C_{μ} and $C_{\gamma 3}$ is inferred similarly from their absence from MOPC 21, giving the order μ - $\gamma 3$ - $\gamma 1$ and (μ - $\gamma 3$ - $\gamma 1$, $\gamma 2b$)- $\gamma 2a$ - α for the locus. Finally, the high levels of $\gamma 2b$ and $\gamma 2a$ sequences in MOPC 21 suggests that those genes are coupled, yielding the order μ - $\gamma 3$ - $\gamma 1$ - $\gamma 2b$ - $\gamma 2a$ - α .

Thus, we infer a unique, self-consistent gene order from the deletions, although physical linkage data is required to establish the order firmly. Some support for the linkage $\gamma 2b$ - $\gamma 2a$ is provided by a variant of the IgG2b producer MPC 11, which produces heavy chains bearing both $\gamma 2b$ and $\gamma 2a$ sequences⁴⁰.

This map does not indicate the orientation of the genes, that is, which strand is transcribed as mRNA. However, the deletions of the $C_{\gamma 1}$ and $C_{\gamma 3}$ 5'-flanking sequences in MOPC 21 and Y5606, respectively, indicate that those sequences map as follows: μ -5' $\gamma 3$ - $\gamma 3$ -5' $\gamma 1$ - $\gamma 1$ - $\gamma 2b$ - $\gamma 2a$ - α . If we assume that the other C_H genes have the same orientation, the orientation of the locus becomes 5' μ - $\gamma 3$ - $\gamma 1$ - $\gamma 2b$ - $\gamma 2a$ - α 3'.

Deletions can account for rearrangement of heavy chain genes

The deletion model postulates excision of all DNA between expressed V_H and C_H genes (Fig. 1). As heavy chain expression is confined to one allele, Honjo and Kataoka³⁷ postulated that deletions were confined to the chromosome(s) from which the expressed heavy chain is derived. In a tetraploid cell, two of the four homologous chromosomes could be active. Hence, the levels of C_H genes predicted in different plasmacytomas on the allelic deletion model³⁷ are as indicated by the dashed lines in Fig. 5. Our data clearly do not support that specific model, because many of the deletions seem to involve all four copies of the genes lying on the 5' side of the expressed gene. The results are, however, compatible with a more general model in which deletions are not necessarily confined to one allele. The critical requirement is that no C_H gene on the 5' side of the expressed C_H gene is retained on an active chromosome. The results from five lines are entirely compatible with such a model, as no deletions are predicted for the IgM producer HPC 76 and all four copies of the genes lying 5' to the expressed gene have been deleted in MOPC 21, MOPC 173, S107 and MOPC 315, and possibly also in Y5606. In MPC 11 at least two copies, and in HOPC 1 at least one copy, of each

gene on the 5' side of the expressed gene are apparently deleted, so those results are not inconsistent with the model.

The gene levels in all except two of the lines show a 'step' or 'staircase' pattern. Such patterns would be expected on the deletion model (Fig. 1), if deletions extend into the C_H locus for variable distances on different homologous chromosomes. The results for HOPC 1 and MPC 11, however, require deletions entirely within the C_H locus, because these lines retain higher levels of C_μ sequences than of subsequent genes. These complex patterns suggest that some deletions have occurred after the transition to tetraploidy, perhaps as an aberration of the normal rearrangement process.

Biological implications of the deletion model

Thus, our results provide strong support for a deletion model for C_H switching. Each IgG- and IgA-secreting plasmacytoma examined, but not an IgM producer, contained deletions involving one or more C_H genes. Loss of chromosomes bearing the C_H locus, or gross alterations of them, would not generate these effects because the deletions do not include the C_α gene and hence must be confined to a very narrow region in genetic terms. It seems unlikely that the deletions are merely a consequence of neoplasia, because a similar analysis of four T- and four B-lymphoid tumour cell lines, as well as a myeloid cell line, has revealed that neither the C_μ gene nor the C_α gene, nor a sequence 5' to the C_{γ_3} gene have been deleted (S.C., unpublished results). The deletions seem to span sizeable segments of the C_H locus, because they often include more than one C_H gene and, at least for C_{γ_1} and C_{γ_3} , large sequences on the 5' side of the genes as well. An IgG1

producer (MOPC 21) contains a deletion which extends relatively near the C_{γ_1} gene, and an IgG3 producer (Y5606) contains one extending close to the C_{γ_3} gene. As these lines retain only one copy of the active genes (Table 1), we suggest that the deletions constitute an integral part of the somatic rearrangement process which forms an expressed VC gene.

The evidence for simultaneous expression on the surface of B lymphocytes of more than one class of immunoglobulin, primarily IgM and IgD²⁸, seems to conflict with the deletion model, because the receptors are apparently synthesized continually⁵⁰ and both μ and δ chains seem to be derived from the same chromosome²⁸. As B lymphocytes do not divide actively⁵¹, however, the conflict may be resolved by longevity of immunoglobulin mRNA. More probably, μ and δ expression represents a special case in which RNA splicing^{31,32} generates both μ and δ mRNAs from a single transcript. Any double producers of other types could be accounted for by a deletion bringing two C_H genes into proximity and creating a single transcriptional unit.

Our results add to the growing evidence^{13,52,53} that somatic rearrangement events in plasmacytomas are frequently not confined to single alleles. This raises two interesting possibilities (see also ref. 13). First, that rearrangement on one homologous chromosome generates a correct VC join while that on the other occurs in a non-functional fashion, thereby providing a mechanism for allelic exclusion. Second, that correct VC joins are made on both chromosomes and that expression is restricted by some other means.

The deletion model makes strong predictions regarding changes in heavy chain expression during ontogeny of a lymphocyte clone. First, the switching of C_H regions would be irreversible. If a clone had switched, for example, from μ to α expression (IgM to IgA), it could not revert to μ expression, once μ mRNA had been degraded. (Conceivably, the homologous chromosome could be activated, but a special mechanism would be required to ensure activation of the equivalent V_H gene.) Second, the order of expression would be tied to the gene order. Whereas any given clone might not express the whole gamut of C_H genes, those genes that were expressed could only be expressed in the sequence defined by the C_H order.

Since submission of this article, we have learned of observations by Rabbits *et al.*⁵⁴, Coleclough *et al.*⁵⁵ and Yaoita and Honjo⁵⁹ leading to conclusions similar to those reached here.

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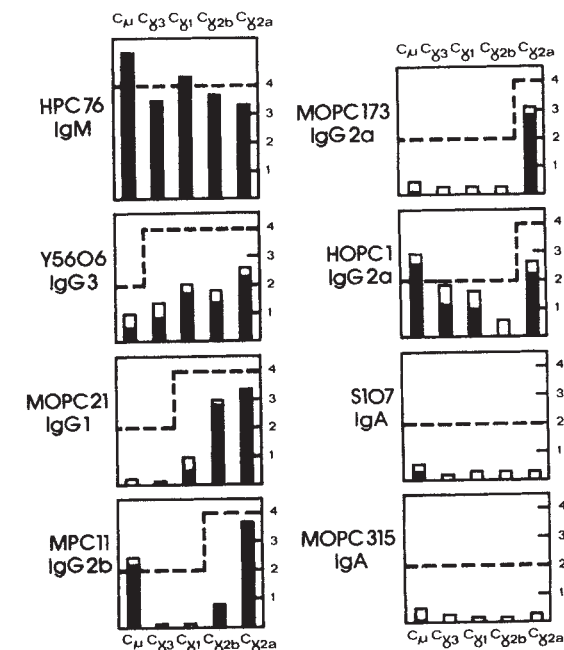


Fig. 5 Number of C_μ , C_{γ_3} , C_{γ_1} , C_{γ_2b} and C_{γ_2a} genes per cell in plasmacytomas synthesizing different classes of immunoglobulin. The copy number of each C_γ gene per tetraploid cell was calculated by multiplying the mean value per haploid genome (Table 1) by 4 and then normalizing to 4.0 copies of the C_α gene per cell (see text). Values for C_μ were determined previously¹³. Because the plasmacytomas were grown as subcutaneous tumours, they are contaminated by non-lymphoid cells¹³. We estimated the levels of C_H sequences contributed by such cells as follows. In MOPC 21 DNA, C_μ and C_γ sequences were undetectable in cells grown in continuous culture¹³, so the level of these sequences in MOPC 21 DNA prepared from solid tumours is a measure of C_H sequences contributed by non-lymphoid cells and was subtracted from values obtained for each of the other C_H genes. Similarly, wherever any C_H gene occurred at <0.3 copies per cell (Table 1), we took that as a measure of contamination. In the case of MPC 11, we assumed that the 0.2 copies per cell of C_μ sequences present in embryonic context¹³ were derived entirely from non-lymphoid DNA. The filled portions of the bars represent the values normalized after allowing for contamination and the entire bars the normalized values ignoring contamination.

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Expression of cloned β -endorphin gene sequences by *Escherichia coli*

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DNA coding for the opiate peptide β -endorphin has been cloned into bacterial plasmids in such a way as to direct the synthesis of a hybrid β -galactosidase/ β -endorphin protein. This hybrid protein can readily be cleaved in vitro to release biologically active β -endorphin.

THERE are two basic approaches to the programmed expression of mammalian gene sequences in bacteria. In one, the cloned mammalian coding sequences are spliced into the bacterial gene such that the bacterial initiation codon is followed directly by the codon for the first amino acid of the mammalian protein¹. In the other, the coding sequence is inserted into an internal position in a bacterial gene so that the sequences are in phase with the bacterial coding sequences, which results in the synthesis of a hybrid bacterial-mammalian protein, from which the mammalian protein has to be released^{2–8}. Although release can be a problem, there may be several advantages to the hybrid protein approach. First, it minimizes the chances of a disturbance of the mRNA secondary structure in the region of the normal ribosome binding site and initiation codon, causing a reduced rate of initiation of protein synthesis. Second, degradation of the mammalian protein in the bacterium may be reduced when it is fused. These factors may increase the yield of the mammalian protein. Third, construction of plasmids for synthesis of a hybrid protein is ordinarily simpler than for exact expression; for instance there may be less need for chemically synthesized DNA^{1,3} with the hybrid protein approach. Finally, for direct expression of an exact protein, the first amino acid must be methionine which may not be cleaved.

We have, therefore, chosen the hybrid protein approach in an attempt to persuade bacteria to synthesize β -endorphin, a 31 amino acid endogenous opioid. This protein is generated by post-translational processing from a larger precursor protein

(ACTH/ β -LPH precursor) which also contains the sequences of corticotropin (ACTH) and β -lipotropin (β -LPH)^{9–17}. We report here the expression, in *Escherichia coli*, of cloned β -endorphin sequences as a hybrid β -galactosidase/ β -endorphin protein and describe the release, from the hybrid protein, of mature, biologically active β -endorphin.

Construction of a plasmid for expression of β -endorphin gene sequences

To programme bacteria to express β -endorphin gene sequences, we used a previously cloned DNA fragment derived by reverse transcription of mRNA coding for the mouse ACTH/ β -LPH precursor protein¹⁸. This fragment contains the coding information for amino acids 44–90 of β -LPH (Fig. 1). This portion of β -LPH is composed of the carboxy-terminal 15 amino acids of β -melanocyte stimulating hormone (MSH) and the complete β -endorphin sequence with the exception of the C-terminal glutamine. It was thus necessary to modify this cloned fragment in order to recreate the codon for the C-terminal amino acid, insert a stop codon, link the fragment in phase to a bacterial gene, and devise a method for the release of mature β -endorphin from the hybrid protein.

Our approach to these problems is outlined in Fig. 2. The cloned fragment, released from the plasmid by *Hind*III digestion, was first cleaved in the β -MSH coding region with *Hpa*II to facilitate later 'phasing' with the bacterial β -galactosidase gene. The single stranded *Hpa*II and *Hind*III termini were then partially 'filled-in' using reverse transcriptase, dATP and dCTP; this recreated the 3'-terminal glutamine codon. The remaining unpaired terminal nucleotides

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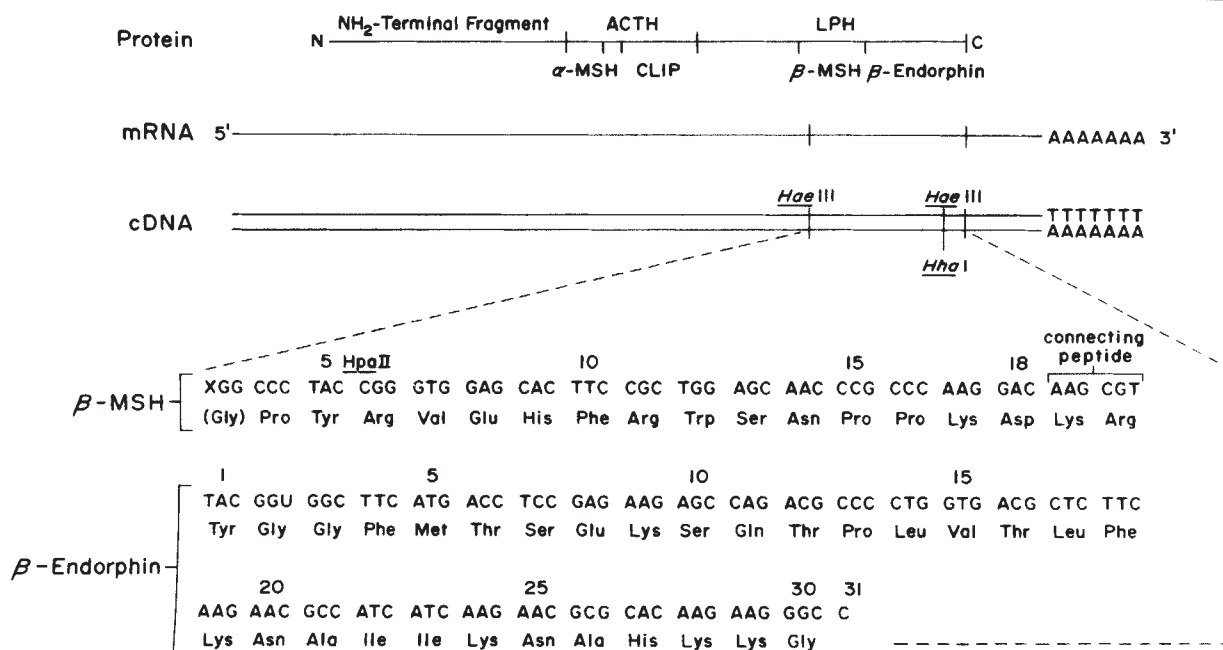


Fig. 1 Nucleotide sequence of the previously cloned fragment of cDNA to ACTH β -endorphin mRNA used to construct a plasmid for expression of β -endorphin sequences (from ref. 18). The plasmid containing this fragment (pMAE-1) had been constructed by ligating chemically synthesized decamers containing the restriction sites for endonuclease *Hind*III to an *Hae*III digest of the cDNA. This fragment was then cloned in the *Hind*III site of plasmid pBR322. The *Hae*III sites on either end of the cDNA are shown, but the *Hind*III 'linker' sequences are not indicated. The protein structure of the ACTH- β -endorphin precursor and its constituent hormones are shown and the amino acids of β -MSH and β -endorphin are numbered. The *Hpa*II endonuclease site at which this DNA was cleaved in the construction of the expression plasmid is also indicated (see Fig. 2).

were removed by *S*₁ nuclease digestion and the blunt-ended fragment ligated to a synthetic octanucleotide containing the recognition site for *Eco*RI. Apart from providing the *Eco*RI termini necessary for ligation to the β -galactosidase gene, this octanucleotide provided a stop codon directly following the 3'-terminal glutamine codon. The modified fragment was then ligated into the *Eco*RI site of the plasmid p β -gal (pBR322 carrying the *lac* control region and the coding sequence for β -galactosidase²). The unique *Eco*RI site in this plasmid occurs at the codon for amino acid 1004 of β -galactosidase. This resulted in the formation of a recombinant plasmid (p β -gal-end) carrying a hybrid β -galactosidase- β -endorphin gene sequence.

Expression of the hybrid β -galactosidase/ β -endorphin gene

E. coli RR1 cells carrying the recombinant plasmids p β -gal or p β -gal-end were examined for expression of polypeptides under control of the *lac* promoter. Polyacrylamide gel electrophoresis of extracts from stationary- or log-phase cells containing p β -gal demonstrated the presence of large amounts of the β -galactosidase peptide, which was absent from uninduced control cells (Fig. 3). This high copy number of this plasmid (30–50 copies per cell) renders host cells mostly constitutive for β -galactosidase synthesis. Similar analysis of cells carrying p β -gal-end demonstrated the absence of the β -galactosidase peptide and the appearance of a new band reflecting a protein approximately 30 amino acids larger than β -galactosidase—the size expected for the β -galactosidase- β -MSH- β -endorphin hybrid protein (Fig. 3e). This protein is also synthesized under the control of the *lac* promoter, as was demonstrated by its induction with isopropyl- β -thiogalactoside (IPTG; Fig. 3f).

Although it accounts for several per cent of the total cellular protein, the expression of the presumptive hybrid protein appears to be reduced in cells carrying the parental p β -gal plasmid as compared to the synthesis of β -galactosidase. However, this level seems to be increased by amplification of

the plasmid DNA sequences (Fig. 3i). As was previously found with both the A and B chains of human insulin linked to the same β -galactosidase fragment³, the hybrid protein is insoluble and can be recovered from a high speed pellet of cell extracts where it represents a substantial proportion of the total proteins (Fig. 3h).

Release and partial purification of biologically active β -endorphin

The steps used to release mature β -endorphin from the presumptive hybrid protein are shown in Fig. 4 and detailed in the legend. To do this, we made use of: (1) the arginine residue that precedes the β -endorphin sequence (Fig. 1), which can be a site for proteolytic cleavage by trypsin; (2) the absence of any internal arginines in this hormone (Fig. 1); and (3) the observation that the lysine residues in β -endorphin (Fig. 1) could be protected from attack by trypsin by reaction with citraconic anhydride *in vitro*²⁵. Thus, after dissolving the precipitated hybrid protein and treatment with citraconic anhydride at pH 9, the β -endorphin (containing the modified lysine groups) was released from the hybrid protein by cleavage with trypsin. Native β -endorphin was subsequently produced by removal of the citraconic groups at pH 3. Cell extracts prepared in this manner were assayed for β -endorphin activity. The β -endorphin was further purified from this preparation with the use of glass extraction as described in Fig. 4 legend.

Immunological activity of partially purified bacterially synthesized β -endorphin

To test the immunological activity of the released β -endorphin, a heterologous radioimmunoassay was designed (Fig. 5). The antiserum had been raised against mouse pro-ACTH/ β -LPH; radiolabelled synthetic human β -endorphin was used as the radioligand. The human hormone differs from that of mouse β -endorphin only at position 27 where a tyrosine is present

Fig. 2 Construction of a plasmid for the expression of β -endorphin sequences by *E. coli*. All recombinant DNA experiments were carried out in accordance with ASCORD (Australia) or NIH guidelines. Recombinant plasmids were isolated from chloramphenicol-amplified cultures as previously described¹⁹. The sources for restriction endonucleases, RNA-dependent DNA-nucleotidyl-transferase (reverse transcriptase), other enzymes and reagents, unless noted otherwise, are provided in previous publications^{5,18,20,21}. The cloned 150-base pair fragment from pMAE-1 (Fig. 1, ref. 18) was isolated by *Hind*III digestion and electrophoresis in a 7% polyacrylamide gel. Digestion of 500 ng DNA with 5 U of *Hpa*II was carried out at 37°C for 1 h in 20 μ l 6 mM Tris (pH 7.5), 6 mM MgCl₂ and 6 mM β -mercaptoethanol. Following phenol extraction and ethanol precipitation, the DNA was dissolved in 50 μ l of a reaction mixture containing 500 μ M each of dATP and dCTP. Incubation was carried out at 37°C with 10 U of reverse transcriptase for 15 min. Unincorporated triphosphates were then removed by chromatography on Sephadex G-50 and after ethanol precipitation, the DNA was dissolved in 50 μ l of 0.3 M NaCl, 30 mM Na acetate pH 4.5, 3 mM ZnCl₂ and incubated with 5 U of *S*₁ nuclease at 20°C for 5 min followed by phenol extraction and Sephadex G-50 chromatography. The duplex DNA was then ligated to a 20-fold molar excess of octamers ('linkers') containing the restriction site for endonuclease *Eco*RI in 40 μ l of 60 mM Tris (pH 7.5), 8 mM MgCl₂, 10 mM β -mercaptoethanol, 1 mM ATP, using 2 μ l of bacteriophage T₄ DNA ligase (New England Biolabs) at 4°C for 24 h. Following digestion with 50 units of *Eco*RI at 37°C for 5 h, the linker fragments were removed by Sephadex G-50 chromatography and the DNA ligated to 1 μ g of *Eco*RI-cleaved, and alkaline phosphatase treated²¹ β -gal DNA², and transformed in *E. coli* RR1²². Plasmid DNA was isolated from 3-ml cultures of several independent recombinant clones and cleaved with *Hae*III to determine the orientation of the cloned fragments. One of these clones (β -gal-end), with the insert in the correct orientation for readthrough of β -endorphin sequences from the β -galactosidase gene, was selected for further analysis. The terminal nucleotide sequences of the inserted fragment were determined by digesting the plasmid β -gal-end with *Eco*RI endonuclease, isolating the inserted β -endorphin gene fragment by gel electrophoresis and electroelution, 3'-terminal labelling of the *Eco*RI termini with [α ³²P]dATP (Amersham, 2,000 Ci mmol⁻¹) and reverse transcriptase, cleaving the fragment with *Hha*I, isolation of the two digestion products by electrophoresis in a 10% polyacrylamide gel, and subjecting these to DNA sequence analysis by the method of Maxam and Gilbert²³. The sequences were found to be identical to those shown.

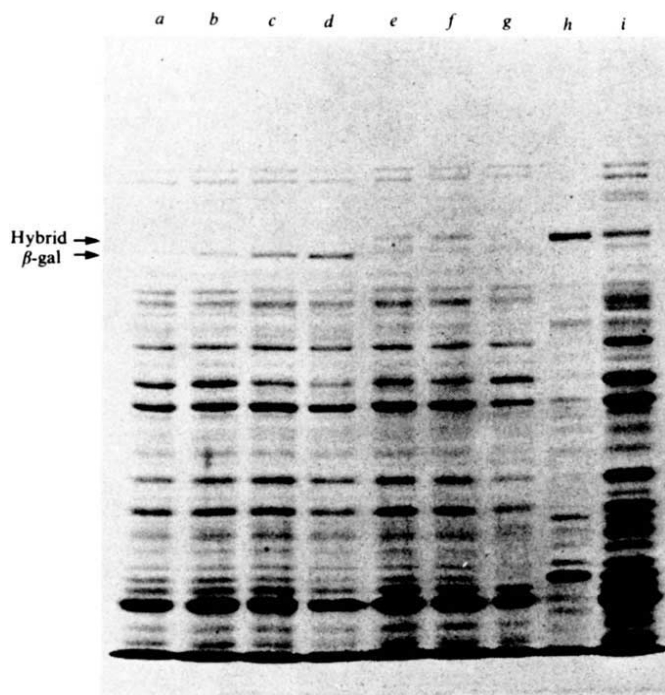
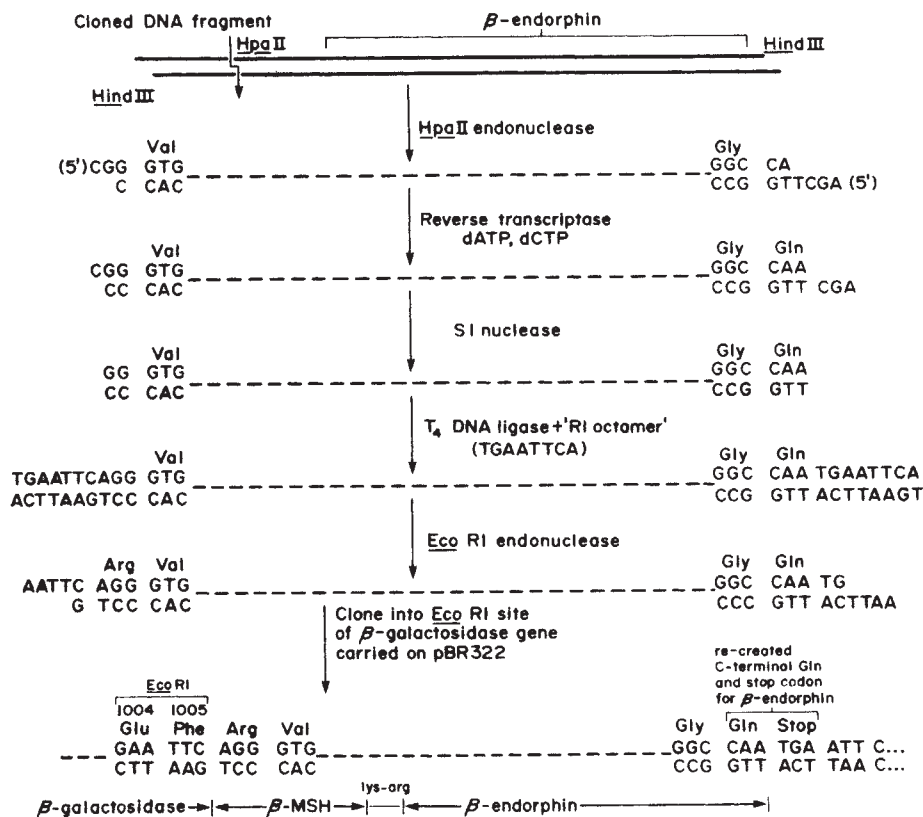


Fig. 3 Proteins synthesized from plasmids β -gal or β -gal-end. Bacteria harbouring β -gal or β -gal-end were grown at 37°C to log phase (A_{650} of 0.25) in L-broth and collected by centrifugation. Cells were dissolved in SDS sample buffer and the equivalent of 50 μ l of original culture subjected to electrophoresis in a 7% polyacrylamide gel and visualized by staining with Coomassie blue²⁴. The positions of the hybrid protein β -galactosidase- β -MSH- β -endorphin and β -galactosidase are indicated by arrows. a, *E. coli* RR1; b, *E. coli* RR1 induced with 2 mM isopropyl- β -thiogalactoside (IPTG); c, *E. coli* RR1 carrying p β -gal; d, *E. coli* carrying p β -gal induced with IPTG; e, *E. coli* RR1 carrying p β -gal-end; f, *E. coli* RR1 carrying p β -gal-end induced with IPTG. Cells obtained in e were collected by centrifugation and the cell pellet resuspended in phosphate-buffered saline. After disruption by sonication, the cell debris and insoluble protein were pelleted by centrifugation at 15,000 r.p.m. for 30 min. g, Supernatant of p β -gal-end cells after sonication; h, pellet from p β -gal-end cells after sonication; i, proteins from p β -gal-end cells after amplification of plasmid DNA in 50 μ g per ml chloramphenicol, followed by 3 h growth in fresh L-broth.

instead of a histidine and at position 31 where a glutamic acid residue replaces a glutamine²⁸. These changes do not inhibit the ability of the human hormone to react to antiserum raised against the mouse hormone. Immunological activity was examined in partially purified extracts prepared as described in the legends to Figs 3 and 4 from fully induced *E. coli* RR1 cells containing the recombinant plasmid p β -gal-end.

The endorphin purified from *E. coli* RR1 cells carrying the recombinant plasmid p β -gal-end competitively inhibited the binding of human ¹²⁵I β -endorphin to the mouse endorphin antibody (Fig. 5). As based on the immunological activity in the glass-purified material and the recovery from the trypsin-treated preparation (see the legends to Figs 4 and 5), it is estimated that 0.5 μ g of released endorphin of molecular weight 3,900 can be obtained from 10⁹ cells (8 $\times$ 10⁴ molecules of endorphin per cell). By contrast, extracts of cells harbouring the plasmid p β -gal which had been purified in a similar manner showed no competition for endorphin antibody binding sites. The activity demonstrated by extracts of these cells (over a series of 11 dilutions to 1:10⁴) was the same as that of the assay buffer.

To determine if the material reactive to antiserum to β -endorphin was of the anticipated size, 60 μ l of the glass bead-purified preparation was filtered on a Sephadex G-50 column (Isolab 3 ml propylene column) and the column fractions were assayed for immunological activity. The column had been pre-calibrated with human β -endorphin²⁸. The profile of immunological activity was included by the column and exhibited an elution pattern identical to the authentic β -endorphin. No immunological activity was found in the excluded fractions. Thus, the released peptide rather than the fusion protein appears to account for the activity.

Biological activity of bacterially synthesized β -endorphin

The opiate activity of endorphin prepared from extracts of fully induced *E. coli* RR1 cells containing the recombinant plasmid p β -gal-end was examined by testing its ability to bind to opiate receptors of rat brain membrane preparations³¹ and to elicit an opiate-like effect of inhibiting the stimulation by prostaglandin E₁ of cyclic AMP accumulation in the neuroblastoma-glioma hybrid cell line NG108-15³².

The receptor-binding activity of the material synthesized in the bacteria was examined (Table 1) by testing its ability to inhibit the binding of a radiolabelled agonist (³H-D-Ala²-Met⁵-enkephalinamide) or antagonist (³H-naloxone). Endorphin prepared according to Figs 3 and 4 legends (without glass extraction) inhibited both the agonist and antagonist binding. As might be expected with this crude preparation (purified material was not available for these experiments), some nonspecific inhibition of binding was observed in the control reaction, where an extract from p β -gal was used; however, this was substantially less than in the reaction with an extract from p β -gal-end.

The neuroblastoma-glioma hybrid cell line NG 108-15 is richly endowed with opiate receptors³² and possesses an adenylate cyclase which can be stimulated by prostaglandin E₁ (ref. 34). This stimulation is inhibited by morphine and the inhibitory effect of morphine can be prevented by naloxone³⁵. Endorphin prepared as described in Figs 3 and 4 legends (without glass extraction) showed a significant inhibition of the stimulation by prostaglandin E₁ of adenylate cyclase activity (Table 1). This inhibition was completely reversed by addition of the opiate antagonist naloxone, and it was much greater than the activity observed in the control reaction (Table 1).

Discussion

This report demonstrates the expression of cloned β -endorphin gene sequences by bacteria in a form from which substantial amounts of biologically active β -endorphin can be obtained.

Table 1 Opiate receptor binding and opiate-like actions of extracts of p β -gal-end

	% Inhibition	
	p β -gal-end	p β -gal
Competition for receptor binding		
³ H-Ala ² -Met ⁵ -enkephalinamide (agonist)	84 (83–85)	65 (63–71)
³ H-naloxone (antagonist)	68 (67–69)	25 (17–32)
Cyclic AMP production		
No antagonist present	23.3 (21.9–24.6)	5.1 (5.0–5.2)
+ Naloxone	0	0

Rat brain membrane preparations were isolated according to the method of Snyder³¹ with slight modification. Male Sprague-Dawley rats (200–220 g) were killed by decapitation and the brains minus cerebellum were homogenized in 40 volumes of 0.05 M Tris-HCl buffer pH 7.7 at 4°C. The homogenates were first centrifuged at 600g for 5 min to remove the nuclei. The supernatant was then centrifuged at 4°C for 15 min at 49,000g. The pellets were suspended in 30 ml (per rat brain) of the same Tris buffer. The resuspended pellets were then incubated at 37°C for 30 min and centrifuged for 15 min at 49,000g. The final pellet from each rat brain was then resuspended in 40 ml of 0.05 M Tris-HCl buffer containing 100 μ g ml⁻¹ of bacitracin and used for the binding assays. The binding experiments were performed, as previously described at 25°C for 20 min, or at 0°C for 3 h. Reaction mixtures contained 1 ml of tissue suspension and 1.5 nM of ³H-D-Ala²-Met⁵-enkephalinamide (New England Nuclear, 38 Ci mmol⁻¹) or ³H-naloxone (New England Nuclear, 25 Ci mmol⁻¹); 4.5 μ g of bacterial extract was incubated in the reaction with ³H-D-Ala²-Met⁵-enkephalinamide and 0.65 μ g was used in the reaction with ³H-naloxone. In each case the extracts were from a separate preparation. Non-radioactive levorphanol or naloxone³¹ was used as standard competitor for each assay. The reaction was terminated by vacuum filtration over Whatman glass fibre filters (GH-B). The filters were washed with a large volume of ice-cold Tris buffer and placed in 12 ml of Hydromix scintillation fluid (Yorktown Res.). In parallel experiments 10⁻⁶ M of naloxone were incubated to estimate the background binding which was subtracted from the total binding to yield the specific binding. Results are reported as per cent inhibition of specific binding that was 5,613 c.p.m. per assay with ³H-D-Ala²-Met⁵-enkephalinamide and 2,869 c.p.m. per assay with ³H-naloxone. Mean values and ranges (in parentheses) of triplicate incubations are shown. To test for biological activity, the neuroblastoma-glioma hybrid cells (line NG108-15) were used³². Cells were grown in T-flasks (one T-75 flask per 60 assays) in Dulbecco's modified Eagle's medium (DMEM)³³ containing 10% fetal calf serum, 0.1 mM hypoxanthine, 10 μ M aminopterin and 16 μ M thymidine. At confluency, the cells were collected and homogenized in 0.32 M sucrose, 40 mM HEPES and 2 mM EDTA, pH 7.6. Incubations^{34,35} were carried out at 30°C for 15 min in a total volume of 100 μ l containing 3 $\times$ 10⁶ c.p.m. of ³²P-ATP, 10 units creatine phosphokinase, 50 μ M prostaglandin E₁ (PGE₁; Upjohn), 5 μ l of opiate agonist or extract (3.5 μ g) of pellet material (prepared from fully induced *E. coli* RR1 as indicated in legends to Figs 3 and 4), 20 mM HEPES, 5 mM MgCl₂, 1 mM cyclic AMP, 20 mM creatine phosphate, 0.1 mM ATP, 0.125 mM phosphodiesterase inhibitor (ZK 62711, Schilling) and 1 mM protease inhibitor (Sigma). The reaction was stopped by the addition of 150 μ l of 1 M HClO₄ and 0.3 ml of H₂O containing ~30,000 c.p.m. of tritiated cyclic nucleotide per tube. After mixing and centrifuging, the supernatant solutions were poured onto Dowex 50 columns. [α -³²P]-cyclic AMP was then separated from other nucleotides by the two-column (Dowex 50 and alumina) method described by White and Karr³⁶. The adenylate cyclase activity, which was stimulated by PGE₁ (level of cyclic AMP released increased from 30 to 180 pmol per mg protein per min) in the absence of opiate agonist or test material was used as control^{33,34}. The activity of the bacterially synthesized endorphin preparation is expressed as the ability to inhibit this stimulation. The agonist activity of the test material was further examined by the reversibility of the effect by the opiate antagonist naloxone³⁴. Mean values and ranges (in parentheses) of triplicate incubations are shown.

These data, along with those recently demonstrating the synthesis of somatostatin², insulin³ and growth hormone^{1,6} show the potential usefulness of bacteria to synthesize large

quantities of polypeptides of medical and economic importance. The methodology used in this report differs from and therefore complements that previously used for the other proteins. In all of these studies in which an exact hormone was produced, fairly large fragments of synthetic DNA were used. In the current study, small fragments of DNA containing restriction endonuclease sites were used to create the proper reading frame, to add the codon for one amino acid and a

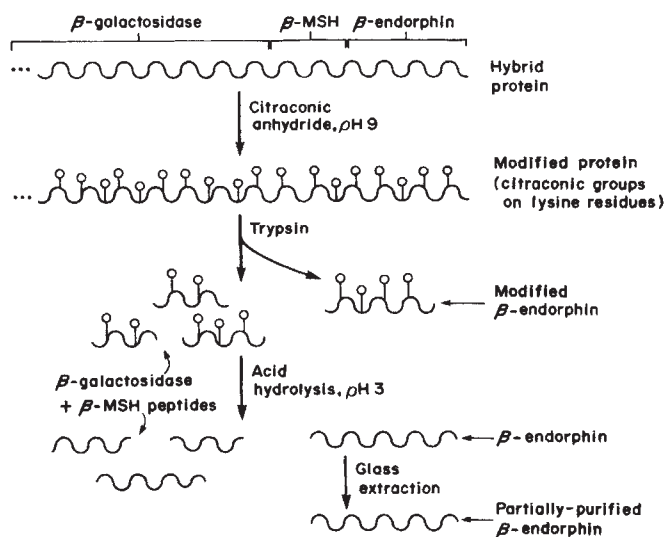


Fig. 4 Release of β -endorphin from the β -galactosidase- β -MSH- β -endorphin hybrid protein. The pellet obtained, as in Fig. 3 was dissolved in 10 ml of 6M guanidinium chloride, 1% β -mercaptoethanol and centrifuged at 20,000 r.p.m. for 1 h. Citraconic anhydride (Fisher) was added to the supernatant in three lots of 10 μ l over a 15-min period, during which time the pH was maintained between 9 and 11 by the addition of 2M NaOH²⁵. The solution was then dialysed overnight against 50 mM ammonium bicarbonate followed by the addition of trypsin (Worthington) to 0.5 mg ml⁻¹ and incubation at 37 °C for 12 h. Trypsin was inactivated by the addition of phenylmethylsulphonyl fluoride (PMSF) to 1 mM for 1 h and the solution made 1% in formic acid before lyophilization. The dried protein was then dissolved in Tris (base) buffer pH 7.6 to yield a protein concentration²⁶ of 0.5–1.0 μ g ml⁻¹. This preparation was tested for immunological and biological activity. To further purify β -endorphin, a modification of the technique devised for the extraction and purification of ACTH by Rees *et al.*²⁷ was used. To 0.5 ml aliquots of the crude endorphin preparation were added 0.5 ml of horse serum (Grand Island Biological) and 50 mg glass powder (140 mesh, Corning Glass Works, washed once with water, heated for 24 h at 120 °C and then stored in a desiccator until use) in a 15-ml plastic centrifuge tube. The samples were vortexed for 30 s and then centrifuged for 5 min at 3,000 r.p.m. The supernatant was discarded and the glass adsorbant washed with 3 ml of water. The endorphin was removed from the glass by addition of 1 ml of 50% acetone in 0.25–5.00 M HCl, vortexing for 30 s and then centrifuging as above. The endorphin-rich supernatant was then transferred to 10 $\times$ 75 mm polystyrene tubes (Falcon) and evaporated to dryness with a fine stream of nitrogen in a water bath at 45 °C. Dried samples were reconstituted with Tris buffer (pH 7.6) and serially diluted for radioimmunoassay. To monitor the recovery of β -endorphin in the extraction procedure, a parallel experiment was performed in which the recovery of ¹²⁵I- β -endorphin was measured. The recovery was 60%. The human β -endorphin had been synthesized by the solid phase technique by Li *et al.*²⁸ and labelled with ¹²⁵I by a chloramine-T procedure²⁹. The iodinated peptide was then purified by adsorption to 35 mg of glass powder (Corning) in 10 ml of buffer (pH 7.4) containing 0.05 M sodium phosphate, 0.25% human albumin and 0.5% β -mercaptoethanol. After mixing for 5 min, the suspension was spun at 3,500 r.p.m. for 5 min and the pellet was washed once with water and then eluted with 2 ml of 40% acetone in 0.25 M HCl. The ¹²⁵I- β -endorphin was then diluted in the phosphate-containing buffer described above before use. Antiserum directed against mouse β -endorphin had previously been generated in rabbits by Allen *et al.*³⁰.

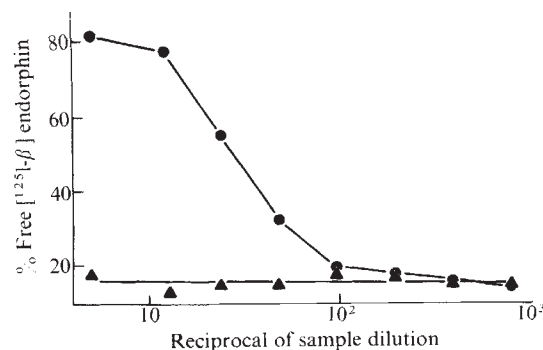


Fig. 5 Immunological activity (competitive inhibition with human ¹²⁵I- β -endorphin for antibody sites) in partially purified extracts from *E. coli* RR1 cells carrying the recombinant plasmid p β -gal-end (●) and p β -gal (▲). Extracts from fully induced bacteria containing these plasmids were prepared as described in Figs 3 and 4 legends. Incubations were carried out at 4 °C for 24 h in a total volume of 150 μ l containing 75 μ l of ¹²⁵I- β -endorphin (10,000 c.p.m.) (prepared as described for Fig. 4), 50 μ l of endorphin antibody (final titre 1:1500 yielded 50% binding; 1:150 yielded 83% binding), 25 μ l of standards or extracts (from lyophilizates at 1 μ g ml⁻¹ protein) at various concentrations in 0.05 M sodium phosphate (pH 7.4), 0.25% human albumin and 0.5% β -mercaptoethanol. Free radioligand was then separated from bound peptides by adding 100 μ l of a dextran-charcoal suspension containing 3% charcoal (Norit A, Pfanstiel Chemical Co), 0.75% dextran (Schwartz-Mann) and 60% horse serum (Grand Island Biological Co.) in 0.05 M sodium phosphate, pH 7.6. After a 5 min incubation the samples were centrifuged at 2,500 r.p.m. for 15 min. The supernatant was aspirated with a glass bent-tip pipette. Radioactivity was counted before incubation (total reaction mixture) and in the charcoal pellet after incubation to calculate the per cent free ¹²⁵I- β -endorphin (ordinate) using human β -endorphin as standard. The assay was most sensitive in the region of 10–100 pg ml⁻¹. Both inter- and intra-assay coefficients of variation were less than 5%.

'stop' codon, but larger pieces of DNA were not required. Thus, even if large pieces of synthetic DNA are not available, it is possible to construct genes to obtain an exact protein.

Bacterially expressed growth hormone^{1,6}, insulin³ and somatostatin² have been shown to have immunological activity but their biological activity has not been reported. By contrast our β -endorphin has biological activity by several criteria. First, it competitively inhibits the binding of an opiate antagonist (naloxone) or agonist (Ala²-Met⁵-enkephalinamide) by the opiate receptors in rat brain membrane preparations. Second, like opiate agonists, the bacterially synthesized material can inhibit the prostaglandin E₁-induced stimulation of adenylate cyclase in cultured neuroblastoma glioma cells. That the material produced by the bacteria had the conformation of authentic β -endorphin was also demonstrated by its ability to react specifically to antiserum to β -endorphin. The immunological activity also eluted from a Sephadex G-50 column in the same region as the standard human β -endorphin, thus supporting the idea that there was cleavage of the endorphin from the hybrid protein.

Were our bacterially expressed β -endorphin to be considered for clinical use (for treatment of pain, diarrhoea or psychiatric problems, for example^{37,38}), it should be noted that it differs from human β -endorphin by two amino acids. That may not affect its activity, but if it did, two plasmid codons could readily be altered to give the correct amino acids. Other codon changes could be introduced to direct the synthesis of analogues of β -endorphin which have useful or different opiate agonist or antagonist activity.

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LETTERS

UV spectra of the twin QSOs 0957+561 A, B

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Observations of the twin QSOs 0957+561 A and B by Walsh *et al.*¹ gave the same redshifts in both the emission line ($z_e=1.41$) and absorption line ($z_a=1.39$) systems and also showed similar spectral characteristics in the strengths of the emission lines, absorption lines and continua. This led to the proposal that they were one object, a double image of which was being formed by an intervening massive galaxy acting as a gravitational lens. More extended observations in the 6,000–6,900 Å range² and in the 4,000–7,000 Å region³ confirm the similarity of the two spectra and show very close agreement (14 km s⁻¹ r.m.s.) between the velocities of the absorption line systems, thus strengthening the lens hypothesis. Other data on the structure of the associated radio sources^{4–6} are also consistent with the above proposition. An intervening galaxy has been looked for⁷ which may act as a gravitational lens: very deep red images were taken of the field using a CCD camera on the 200-inch Palomar telescope and supplemented by multichannel spectrophotometric scans of the double system. An elliptical galaxy of red magnitude 18.5 was detected which is nearly superimposed on the south (B) component of the twin QSO and which is the brightest member of a rich cluster of galaxies. The spectrophotometric data of the system show a contribution from this intervening galaxy and from the location of the characteristic Ca II 3,950 Å break, a redshift of 0.39 is estimated. Young *et al.*⁸ conclude that Q0957+561 A, B could be two images of the same object produced by this galaxy but that the lens properties are somewhat complex due to 'aberrations' induced by the rest of the cluster. We present here the first UV observations of Q0957+561 A, B and discuss them in terms of the lens hypothesis.

Two UV spectra of 0957+561 A, B were obtained with the long wavelength spectrograph (LWR) of the IUE. The images of the quasars were centred in the 10×20 arc s entrance aperture and the details of the observing programme are given in Table 1; the spectrum of HD237844, a B3 ($m_v=9.4$) star was taken for comparison.

A strip of data, 20 pixels wide, along a line parallel to the spectra of 0957+561 A, B was extracted from both the RAW unprocessed image and the GPHOT image which is corrected for the intensity transfer function of the LWR camera. The RAW data and the photowrites of the images were examined to identify cosmic ray events and these signals were traced to the GPHOT images, and the enhanced data points were smoothed to the average values in the surrounding pixels. In the GPHOT data the spectra of 0957+561 A, B were confined to a strip of ~7 pixels wide and were blended. The data were corrected for background by smoothing the signals in 3-pixel wide strips at the edges of the 20 pixels wide band and were then averaged to obtain spectra with an improved signal-to-noise ratio. The spectra were deconvoluted by using the profile

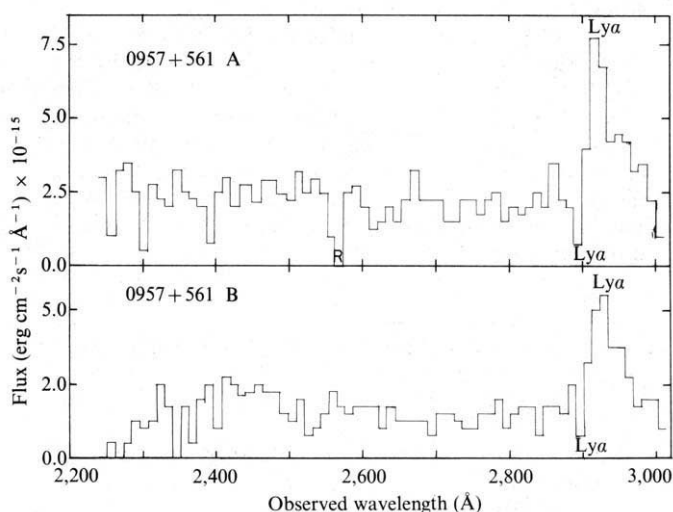


Fig. 1 The UV spectra of 0957+561 A, B binned in 10 Å bands. The Lyα line is present in emission and absorption.

Table 1 Observing log

Object	$\alpha(1950)$	$\delta(1950)$	Image	Integration time (min)	Date
0957 + 561 A, B	09 h 57 min 57.3 s	+ 56° 08' 18"	LWR 6472	347	24 Dec 1979
0957 + 561 A, B	09 57 57.3	+ 56 08 18	LWR 6473	398	25 Dec 1979
HD 237844	09 48 31	+ 55 57 38	LWR 6471	3	24 Dec 1979

of the point-spread function perpendicular to the direction of dispersion obtained from the spectrum of HD237844. As the IUE spacecraft and the scientific instrument parameters were similar when the three spectra were obtained, this procedure seems justified. The deblended spectra of Q0957 + 561 A, B binned in 10 Å bands are shown in Fig. 1.

The UV spectrum in Fig. 1 covers the rest-frame wavelength range 950–1,250 Å. A continuum is present together with the Ly α 1,216 Å line in emission and absorption at redshifts consistent with those given by Walsh *et al.*, that is 1.41 and 1.39 respectively. Table 2 gives the absolute intensities of the Ly α emission line in each spectrum together with its emission and absorption equivalent widths. These latter values are not unusual, lying within the range typical of QSOs. In these weakly exposed spectra, the absorption equivalent widths are the least accurate but give an equivalent hydrogen column density $N(H)$ of $\sim 4 \times 10^{19} \text{ cm}^{-2}$. From their visible spectra, Wills and Wills³ find the corresponding value for $N(\text{Fe}^+) \sim 3 \times 10^{14} \text{ cm}^{-2}$. Assuming Fe is mainly in the singly ionized state and normal cosmic abundances gives $N(H) \sim 10^{19} \text{ cm}^{-2}$. Taking the uncertainties involved into account, these two numbers are probably not inconsistent with each other.

Table 2 Observed intensities and equivalent widths of emission and absorption Ly α *

	0957 + 561 A	0957 + 561 B
Intensity of emission Ly α ($\text{erg cm}^{-2} \text{ s}^{-1} \times 10^{-14}$)	4.4	3.5
EW of emission Ly α (Å)	140 (58)	154 (64)
EW of absorption Ly α (Å)	13 (5)	9 (4)

*Rest-frame values in brackets.

The ratio of the UV fluxes of QSO B to A has been derived by binning the data over the range 2,750–3,000 Å where the signal-to-noise ratio is best. The result and its attendant error is given in Table 3 together with the corresponding visible and radio data taken from the references cited. The flux ratio of QSO B/A is then plotted in Fig. 2 using our UV data with the visible data of Walsh *et al.*¹

The variation and structure of the flux ratio in the visible, which departs from the constant value predicted by the lens hypothesis, led Walsh *et al.*¹ to suggest that differential extinction in the two light paths around the lensing galaxy causes a reddening of component B relative to A with the upturn near 5,300 Å being produced by a redshifted 2,200 Å feature. This hypothesis has been investigated by Wills and Wills³ whose data gave a best fit for a differential visual extinction $A_v = 0.65$ at a redshift $z = 1.2$. This requires QSO B

to be intrinsically brighter than A by a factor of ~ 2.5 which opposes (on the lens theory) the ratio value of 0.74. This may be explained by variability in the source, either due to the time delay of the order of months between the two images, or to different variability at optical and radio frequencies. However, Wills and Wills³ do not detect any optical variability and conclude that the alternative hypothesis of two separate objects cannot be ruled out.

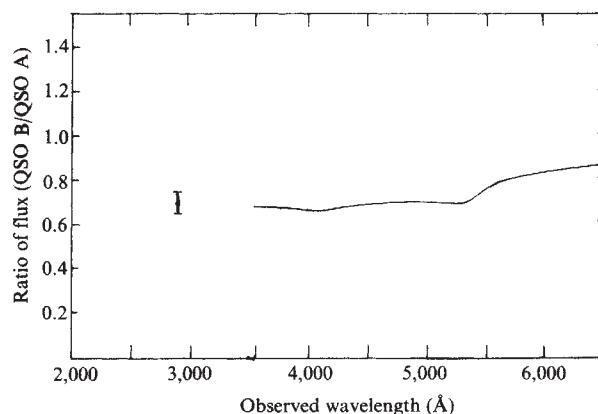


Fig. 2 The ratio of fluxes from 0957 + 561 B to fluxes from 0957 + 561 A. The visible data are from ref. 1 (solid line); I, present data.

The UV data presented here allow a far better test of any differential extinction effects, first because any extinction will be greater in the UV but, more important, any contribution to the observed UV fluxes by the intervening elliptical galaxy⁷ will be negligible, unlike the visible region. Indeed, Young *et al.*⁸ propose that the upturn near 5,300 Å is the characteristic Ca II break caused by the contribution of the intervening galaxy and, in support of this, we propose that the less conspicuous feature near 4,000 Å (see Fig. 2) is the corresponding Mg II break. We can therefore make a comparison between the UV and radio fluxes to derive a direct estimate of any differential extinction. The ratio of the flux ratio in the two frequency ranges is

$$\left[\frac{B}{A} \right]_{\text{UV}} / \left[\frac{B}{A} \right]_{\text{radio}} = 0.92 \pm 0.07$$

Two conclusions are drawn from this result. First, on the assumption of a galactic extinction law⁹, an upper limit (3σ) can be placed on the differential reddening of $E(B - V) < 0.04$. This low value almost certainly means that the two light paths are almost extinction free, either because the intervening galaxy is dust-free or the light paths are through its dust-free halo. Second, a ratio of unity for the UV and radio flux ratios is in keeping with one of the most fundamental predictions of the gravitational lens theory, and therefore provides strong evidence in favour of that hypothesis.

We thank Drs A. Heck and N. Argue for assistance, also Drs R. F. Carswell and D. Walsh for the ratio of fluxes from the two QSOs as computed from their ground based data. P.M.G. acknowledges useful discussions with Dr R. F. Carswell.

Table 3 Ratio of fluxes from 0957 + 561 B to 0957 + 561 A

Wavelength range (Å)	Ratio	Refs
2,750–3,000	0.72 ± 0.05	Present data
3,500–4,500	0.69	1
4,100–4,700	0.71	3
6,100–7,000	0.76	8
Radio	0.78 ± 0.05	4–6

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Evidence for highly processed material ejected from Abell 30

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The discovery of compact knots of highly processed material apparently ejected from the central star of the emission nebula Abell 30 is reported here^{1,2}. This emission nebula possesses other unusual features. The extended emission region, ~ 2 arcmin in diameter has a very low surface brightness and exhibits a high degree of circular symmetry. The central star 05 fep, $V = 14.3$ is remarkable for its very high temperature of the order 2×10^5 K, as indicated by the strong broad O VI, He II and C IV emission lines in its spectrum and the strong [Ne V] emission in the embedding nebulosity³. Furthermore the star seems to be embedded in a resolved (~ 30 arcs) source of IR emission⁴. These new observations emphasize the peculiar nature of A30 and its importance in studies of stellar evolution.

The central star is located at 08 h 44 min 03.55 s, $+18^\circ 03' 48.2''$ (1950.0) and there is a 17.0 mag companion⁵ at 5.3" and PA 142° . The compact nebulosities were discovered using a combination of a triple exposure three colour plate

taken with the Palomar 48-inch Schmidt and a pair of objective prism plates taken with the UK 48-inch Schmidt with the dispersion north-south and east-west. Figure 1a shows an enlargement of the three colour plate, the images from left to right having effective wavelengths of 6,500 Å (red), 5,000 Å (green-yellow) and 3,600 Å (UV) respectively. The compact nebulosities can be seen as prominent patches of nebulosity surrounding the green-yellow image; they lie within a diameter of ~ 20 arcs around the central star, roughly the same size as the IR source. Of the four obvious features labelled 1 to 4, feature 1 is the galactic star probably unrelated to the planetary nebula. The nebulosities are features 2 to 4, which are absent in the red. The objective prism spectrum in Fig. 1b, which covers the wavelength range 3,400–5,300 Å, shows that the emission from these nebulosities is dominated by lines around 5,000 Å and the north-south dispersed plate shows that nebulosity 3 is similar. The natural conclusion is that the strong line is [O III] $\lambda 5,007$ but the failure to detect the nebulosities in the red then suggests that the Balmer emission must be unusually weak.

Spectra of nebulosities 2 and 4 at a resolution of ~ 5 Å were obtained in January 1979 with a 7×2 arcs aperture using the image photon counting system on the Anglo-Australian telescope. The sky subtracted, in reducing these spectra was an average over several adjacent regions within the smooth extended nebulosity. This corrects the spectra not only for night sky emission but also for any contribution from the extended emission region. A spectrum of the south-east nebulosity 2 after sky subtraction and normalization is illustrated in Fig. 2. Apart from the [O III] doublet the strongest lines visible are He II $\lambda 4,686$, [Ne III] $\lambda 3,869$ and [Ne V] $\lambda 3,426$. The relative intensities of these lines are similar to those normally found in high excitation planetaries. However, there is no evidence of any H β emission although this line is usually more intense than He II 4,686. The spectrum of the north-west nebulosity is similar except that the $\lambda 4,720$ line is comparable in intensity to He II 4,686. Adding together the spectra of the two nebulosities sets a limit to the He II/H He II/H β ratio of $I(4,686)/(H\beta) \geq 20$.

As both the He II 4,686 and H β lines are recombination lines their ratio is directly related to the He²⁺/H⁺ ratio through a ratio of atomic constants and their observed lower limit sets the limit He²⁺/H⁺ ≥ 2 (ref. 6). After applying a small correction for singly ionized helium using the approximation He²⁺/H⁺ = Ne⁴⁺/Ne²⁺ = $0.8I(3,426)/(3,869)$ (refs 7, 8) this sets

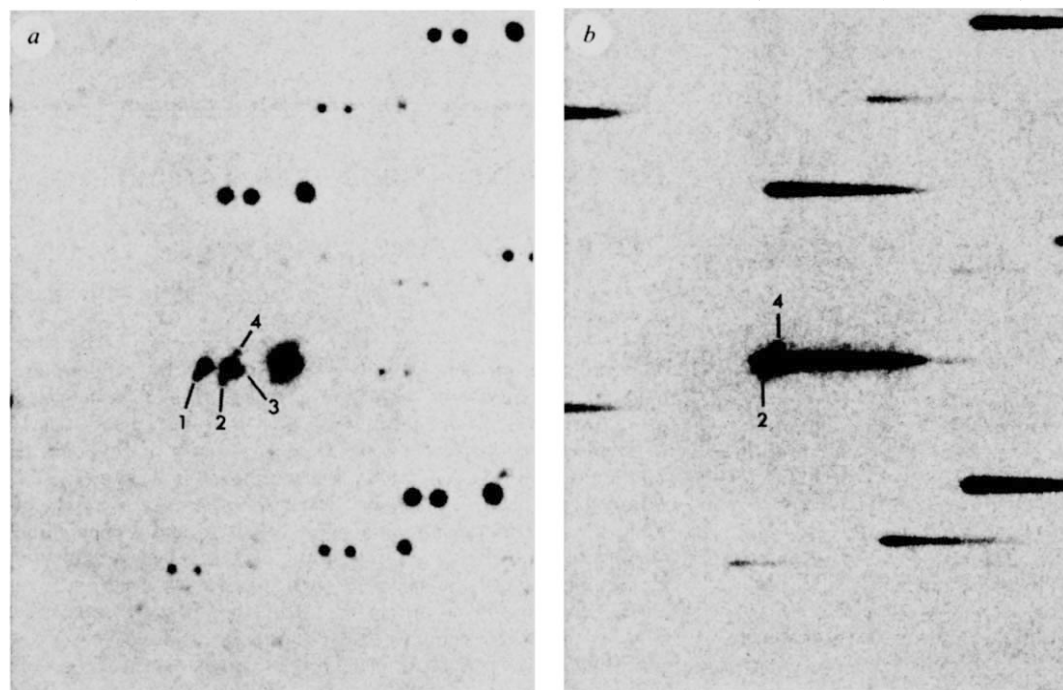


Fig. 1 *a*, An enlargement of the triple exposure three colour plate of A30 taken with the 48-inch Palomar Schmidt. The colours from left to right are, red, green-yellow and UV respectively. Object 1 is a star while features 2 to 4 are the compact nebulosities discussed in the text. The faint extended circular nebulosity is faintly visible. *b*, A reproduction of an objective prism spectrum of A30 taken with the 48-inch UK Schmidt with the dispersion east-west. The emission features marked are the strong [O III] lines from the nebulosities 2 and 4. Fainter emission lines are just visible in the spectrum of the NW nebulosity.

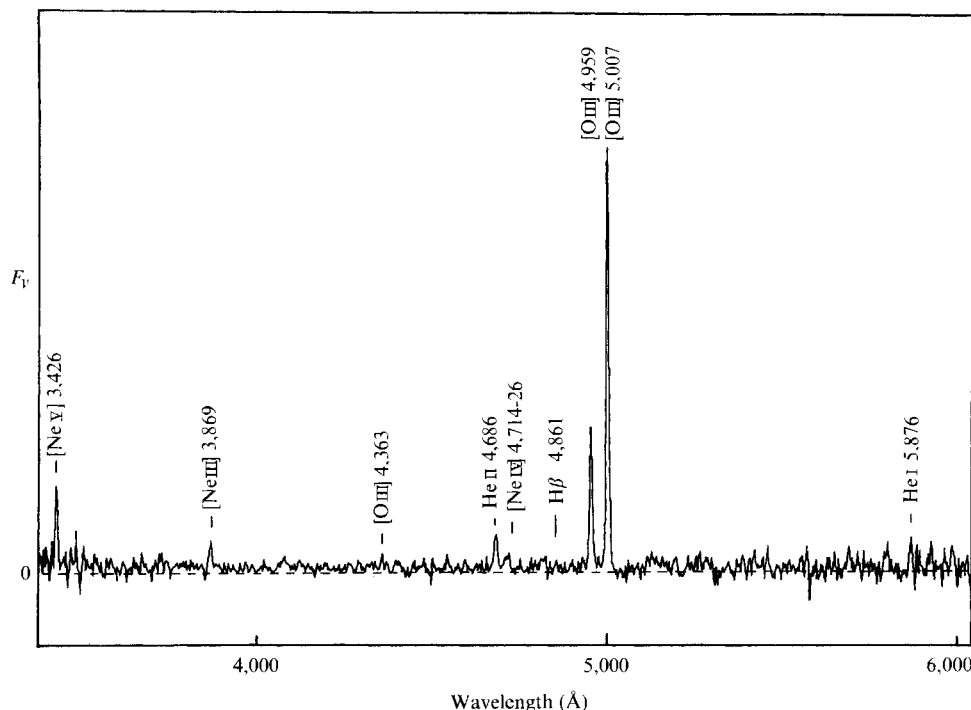


Fig. 2 A spectrum of the southeast nebula, feature 2 in Fig. 1, using a slit size 7×2 arcs at a resolution of ~ 5 Å. Sky subtraction was performed using an average of observations in the extended emission region ~ 20 arcs from the compact nebula. No nebular lines apart from very weak $[\text{O III}]\lambda 5,007$ and $\lambda 4,959$ were present in these background scans.

the limit to the total helium to hydrogen ratio, $\text{He}/\text{H} \gtrsim 2.4$ which is to be compared with a value of ≈ 0.1 in normal planetaries. This result indicates a remarkable enhancement of helium relative to hydrogen in the nebulosities. Wide slit spectra of the extended emission show it to have a more normal composition with $\text{H}\beta$ clearly present but the data, which have a low signal-to-noise ratio do not preclude some enhancement over the solar value.

We have made some preliminary model calculations to investigate the properties of hydrogen deficient nebulae and to study the abundances of some heavy elements: these will be discussed in detail elsewhere. Here we merely point out that the model calculations show that the observed spectrum can be reproduced reasonably well on the assumption: (1) that the filaments are photoionized by radiation from the central star; (2) that the observed He/H ratio arises through these ejecta having been through hydrogen burning which has depleted the hydrogen by a factor of $\gtrsim 20$ with a consequent increase in the helium content; and (3) that the heavy elements have normal relative abundances. The only significant discrepancy between the model and the observations is that the iron lines are predicted at about five times the observed upper limits but iron depletion has been reported in other planetary nebulae⁹ which may indicate a process which selectively removes the iron nuclei from the ejected material.

As a result of both our simple estimate of the He/H ratio and the model calculations, it is established that hydrogen in the compact nebulosities has been depleted by a very large factor. A depletion of hydrogen by a factor of $\gtrsim 20$ indicates an almost complete conversion of hydrogen to helium. Evidently A30 is at a more advanced evolutionary stage than the common planetary nebulae and the central star having already shed its hydrogen envelope to expose the helium core is now shedding its helium envelope. The very tenuous nature of the hydrogen-rich extended nebula is consistent with a model where the previously ejected hydrogen envelope is now almost dissipated. The central star is, therefore, at a very interesting and possibly short-lived phase on evolutionary track perhaps just before collapse to a white dwarf. An alternative view is that we are dealing with a close binary system of helium stars where mass transfer is now taking place. The apparent distribution of the ejected material may give some support to this hypothesis.

Further work on this object is in progress and should help to clarify the evolutionary state of the central star. These more detailed studies should provide an important test of theories of very late stages of stellar evolution.

We thank J. Truran for helpful discussions.

Note added in proof: Since submission of this letter we have become aware that Jacoby¹⁰ has reported an independent discovery of the nebulosities around Abell 30 from photographs taken in $[\text{O III}]\lambda 5,007$.

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The 1978 New South Wales fireball

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A very large meteor fireball passed through the atmosphere above the east coast of New South Wales early in the morning of 7 April 1978. It was seen at 04.44 AEST (18.44 UT, 6 April) by hundreds of people from the cities of Sydney and Newcastle who deluged the news media with telephoned sightings. The resulting publicity led to the collection of many reports, of which 19 were useful in defining the trajectory and ground track of the fireball. I define here the ground track, which is shown in Fig. 1, and include the locations of the eye-witnesses.

An initially puzzling feature of the fireball was the consistently low estimate of the altitude resulting from the combination of observations. This indicated that the fireball should have been visible as it passed overhead at sites 300–

400 km to the south-west of Sydney. Only one newspaper report of a sighting from Mittagong, 80 km south-west from Sydney, was noted. A check with the Australian Bureau of Meteorology disclosed that the inland slopes and highlands of New South Wales at the time were shrouded by stratus cloud and/or fog: the weather station at Bowral, 90 km south-west of Sydney, reporting thick fog at 17.00 UT and continuing at 20.00 h. This was confirmed by a high-resolution IR-image transmitted by the Japanese Geostationary Satellite at 18.00 h UT (on 6 April). The coastal region and inland areas well to the north of Sydney were quite clear.

Sunrise on 7 April occurred in Newcastle at 06.12 h and Sydney at 06.15 h AEST. At the time of passage of the fireball the Moon was below the horizon.

From the reported observations, the New South Wales fireball exhibited the following characteristics:

Time,	04 h 44 ± 1 min AEST (18 h 44 min GMT 6 April)
Brightness,	-16 ± 2 mag at maximum
Heading,	65° ± 5° true
Entry angle,	8° ± 3°
End height,	15 ± 10 km
End point,	152°10' ± 30'E; 32°40' ± 20'S
Velocity,	between 15 and 30 km s ⁻¹

At an early stage it became evident that any resulting meteorite(s) would have descended into the sea at least 50 km offshore. Many of the eye-witnesses reported that the fireball appeared to explode just after crossing the coast, ejecting several short-lived fragments.

The estimate of brightness represents a peak value around the time of the explosion. Many of the witnesses were briefly blinded by it, comparing the luminosity to that of car headlights directly ahead, while others reported the effect in

Table 1 Comparison of two fireballs

Fireball	6 April 1978	25 April 1969
Velocity (km s ⁻¹)	15	20
True radiant RA	155°	163°
True radiant dec.	-14°	-20°
Semi-major axis	1.9 AU	3.6 AU
Eccentricity	0.512	0.761
Perihelion distance	0.935 AU	0.858 AU
Inclination	6.71°	11.35°
Argument of perihelion	36.5°	47.9°
Longitude of ascent node	196.2°	196.2°

terms such as: "There was a blinding flash and the place (the backyard) lit up brighter than the brightest daylight." In subsequent interviews all who witnessed the explosion insisted that the fireball luminosity was more comparable to that of the Sun rather than the full Moon. After making due allowance for the shock to dark-adapted vision, the estimated magnitude of -16 is reasonable and indicates a fireball mass of 5 tonnes or more, depending on the velocity.

As is usually the case when precise timings are not forthcoming, the estimation of velocity proved extremely difficult. In re-enactments during subsequent on-site interviews many of the eye-witnesses indicated angular speeds representing velocities of 30 km s⁻¹ or higher although a few went to the other extreme. The low end-height, which can be more reliably estimated, indicates a velocity of 20 km s⁻¹ or less.

Velocity is the measure to which the orbital elements are most sensitive. Accordingly, the orbit of the fireball has been computed for two reasonable velocities and is compared in Table 1 with a similar set of elements derived by Hindley and Miles¹ for another large fireball which passed over the British Isles in April 1969.

The orbit is direct and of a low inclination typical of those usually derived for meteorite-producing fireballs. The similarity with the 1969 fireball is strong. Hindley and Miles drew attention to the close similarity between the 1969 fireball and a 1962 fireball over New Jersey (US) on 24 April 1962 reported by Olivier². It seems probable that all three fireballs resulted from parent bodies having a common origin, either asteroidal debris or a disintegrated nucleus of a short-period comet as suggested by Hindley and Miles.

A notable feature of the 1978 fireball was the high number of witnesses reporting hissing, humming, swishing or crackling sounds simultaneous with the passage of the fireball: these were mentioned in 10 of the 22 high quality reports and 15 of the full total of 33 written reports describing the fireball. Similar sounds accompanied the 1969 fireball according to Andrews *et al.*³, but the actual number is not given. Olivier did not mention anomalous sounds from the 1962 New Jersey fireball, which was only of magnitude -10.

The reports of anomalous sounds from the 1978 fireball resembled many such reports compiled in an extensive survey made by Romig and Lamar⁴. This convinced me that the sounds were physically real and led to a detailed evaluation of all of the information provided by the witnesses. Many significant clues emerged which, when interpreted in conjunction with other fireball reports, narrowed the spectrum of the electromagnetic radiation emitted by the fireball to the very low frequency (VLF) spectral range. Various generation mechanisms known to produce such radiation from nuclear weapons and the aurora were investigated and found inapplicable to meteor fireballs. Recent work by ReVelle⁵ has enabled me to propose a mechanism whereby the enormous power ($\approx 10^{11}$ W) in the fireball wake is converted to electromagnetic radiation at high audio frequencies by the magnetic flux trapped in the highly turbulent wake plasma: this work will be published elsewhere, as will details of some laboratory tests just completed indicating that the E-field

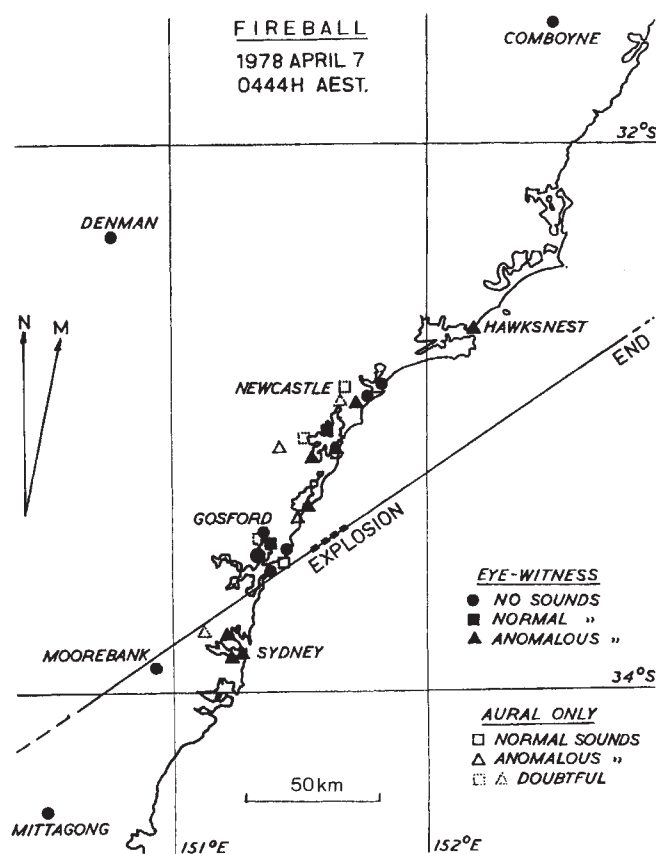


Fig. 1 Track of the New South Wales fireball. Locations of the eye-witnesses are marked.

component of intense VLF radiation generates surface acoustic waves or physical vibrations which are heard by persons with sensitive high frequency hearing response. The transduction of energy from electromagnetic to acoustic, therefore, depends on the nature of the materials and objects close to the hearer, thus accounting for the sporadic distribution of reports of anomalous sounds from fireballs. Sounds emanating from brilliant auroral displays are similarly explicable.

I thank A. A. Griffin, NRC of Canada, for assistance in computing the fireball orbit.

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Muon catalysis for energy production by nuclear fusion

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In 1957 Jackson¹ considered the possibility of using muon-catalysed dt (deuterium-tritium)-fusion for energy production. His conclusion was negative. However, the situation has now changed and this problem should be reconsidered. The suggestion that muons would be suitable as catalysts of dt fusion was first made by A. D. Sakharov and Ya. B. Zeldovich (see ref. 2). It is shown here that the use of the muon catalysed dt fusion combined with the fissile nuclides blanket can provide a positive energy gain.

Basic reactions of muon catalysis are shown in Fig. 1. When slowing down, a negative muon forms the mesic atoms ($d\mu^-$) or ($t\mu^-$) with probabilities c and $(1-c)$, proportional to the relative concentration of deuterium and tritium in the dt mixture. During the time τ_{dt} the muon is transferred from deuterium to tritium. During the time $\tau_{dt\mu}$ a mesic atom ($t\mu^-$) collides with deuterium and forms a mesic molecular ion ($dt\mu^-$). It takes a short time τ_f for an exothermic nuclear fusion reaction $d+t \rightarrow {}^4\text{He} + n$ to occur. When the probability is w_s , the muon is captured by ${}^4\text{He}$ and stays there until it decays, alternatively when the probability is $1-w_s$ the muon slows down during the time τ_a and again catalyses the fusion.

The muon spends most of the cycle within mesic atoms. The fusion time [which is small because of the existence of a nuclear resonance: $\tau_f \sim 10^{-12}$ s (ref. 2)] and the time of muon slowing down and capture [$\tau_a^0 \sim 10^{-10}$ s (ref. 1)] are negligible compared with the muon lifetime ($\tau_0 = 2.2 \times 10^{-6}$ s). [The density dependent quantities τ_a , τ_{dt} and $\tau_{dt\mu}$ with superscript 0 correspond to the liquid hydrogen density $4.25 \times 10^{22} \text{ cm}^{-3}$.]

The probability w_c that the muon quits the cycle is the sum of three quantities each being the product of the probability for the corresponding mesic atom formation and the probability of muon decay inside this atom (τ_{dt} , $\tau_{dt\mu} \ll \tau_0$):

$$w_c = c \times \frac{\tau_{dt}}{\tau_0} + \frac{\tau_{dt\mu}}{\tau_0} + w_s \quad (1)$$

The number of cycles which a muon has time to catalyse is $x_c = 1/w_c$.

According to theoretical estimates $w_s \lesssim 10^{-2}$ (refs 1, 2) and $\tau_{dt}^0 \sim 5 \times 10^{-9}$ s (ref. 3). Recently Ponomarev *et al.*⁴ have shown theoretically that $\tau_{dt\mu}^0 \lesssim 10^{-8}$ s; therefore it is also small compared with τ_0 . Because of a weakly bound excited state [$\epsilon = 0.7 \pm 0.1$ eV with respect to decay into ($t\mu^-$) (ref. 5)] existing in the mesomolecular ion ($dt\mu^-$)⁺ the big excited mesic molecule [$(dt\mu^-)^+ d \ 2e^-$]^{*} is formed through resonance. [A similar mechanism for the formation of ($dd\mu^-$)⁺ was considered previously on theoretical grounds by Vesman⁶ and discovered experimentally by Dzelepov and his collaborators⁷.]

The recent experiments confirm small theoretical values in equation (1): $\tau_{dt}^0 = (3.7 \pm 1.2) \times 10^{-9}$ s and $\tau_{dt\mu}^0 < 10^{-8}$ s (ref. 8). Thus it seems that $w_c \sim 10^{-2}$, that is, one muon can catalyse about $x_c \sim 10^2$ fusion reactions and release ~ 2 GeV of energy^{4,5,16}.

The muons are produced⁹ during the decay of pions generated in the collisions of fast nucleons with nuclei. At moderate energies, $T_0 \sim 1$ GeV per nucleon, π^- are formed mainly in neutron-neutron collisions. The fraction of π^- produced in a single inelastic nn-collision is $w_{nn} = 0.83$; correspondingly $w_{np} = 0.23$ and $w_{pp} = 0$. For this reason, neutron-enriched nuclei should be used as target as well as projectile. Let the average number of π^- produced in a single inelastic collision of nucleons, be $\langle n_- \rangle$. It can be obtained as the average of w_{nk} over the fractions of nn- and np-collisions c_{nk} :

$$\langle n_- \rangle = c_{nn} w_{nn} + c_{np} w_{np}; \quad c_{nn} + c_{np} + c_{pp} = 1 \quad (2)$$

Because of zero total isospin of initial particles in dd-collisions π^- , π^+ and π^0 are produced with equal probabilities and $\langle n_- \rangle = 1/3$. For tt-collisions $\langle n_- \rangle$ increases up to 0.5 according to equation (2).

The yield of pions per unit energy of the incident beam is $Y_\pi = \langle n_- \rangle F T_0^{-1}$, where F is the number of inelastic collisions. In a thin and long cylindrical target, oriented along the beam direction only one collision will occur. For $T_0 = 1$ GeV per nucleon the fraction of inelastic collisions will be $F = 0.5$. By increasing the thickness of the target one can increase the value of F up to $F = 2/3$ without excessive absorption of the pions produced. On the other hand the maximum value of $\langle n_- \rangle$ decreases when beams (d) and targets (Li, Be, D_2O) other than tritium are used. Additional losses of mesons will take place in a converter where π^- are held by the magnetic field until their decay, and also in the walls of the mesocatalytic synthesizer. An optimistic estimate of the conversion coefficient is $\phi = 0.8 \pi^-/\mu^-$. As a result the energy necessary to produce one μ^- is $\epsilon_\mu = Y_\mu^{-1} = (Y_\pi \phi)^{-1} = 5$ GeV per μ^- (ref. 9).

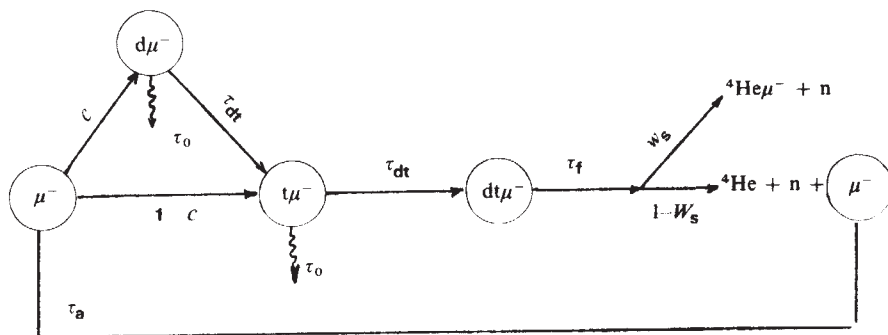


Fig. 1 Scheme of mesocatalysis.

output should be the same, since the values of ξ are close to each other (see Table 1). The methods differ in the values of k_s and α (the fraction of the electric energy, spent on the feeding of the accelerator, or the injector for the TN reactor). The accelerator power required and the value of α for the MC hybreeeder seem to be approximately half those required for EN breeders. Compared to the TN hybreeeder (for example, the project¹⁰ using the Tokamak with the magnetic confinement and with the plasma energy multiplication coefficient $Q=4$), the value of α is four times larger for the MC reactor. However, the difference in their efficiencies is too small (η_s 0.34 and 0.30, respectively) to be considered significant. The difference in the design and the capital costs may seem more important. An evident advantage of the MC hybreeeder is the absence of hot plasma.

The methods listed in Table 1 produce several times more commercial plutonium per fission than the fast-neutron breeder reactors, having $\xi < 0.5$ Pu/fis. Therefore, they are much more effective for the feeding of the cheap thermal neutrons reactors reducing the capital costs per unit energy output of the system. The thermal reactors use the slightly enriched nuclear fuel and made it possible to avoid the external fuel reprocessing cycle. This reduces the risk of nuclear weapons proliferation. However, it is important to remember that only fast-neutron reactors are in current use.

The above estimates show that the system including the MC hybreeeder and usual thermal neutrons reactors provides a gain in electric power. The accuracy of the present estimates is limited, but even a considerable reduction in some of the system parameters (Q , for example) would not change this main conclusion. As to the ability of the MC hybreeeder to compete with the other systems, this depends on the experimental verification of the theoretical estimate of x_c . This estimate is based on the value of w_c , which could be less than 10^{-2} (for example, due to the stripping of μ^- while slowing down $^4\text{He}\mu^-$), and on the value of $\tau_{dtu}^0 < 10^{-8}$ s which is known now only by the order of magnitude. If $x_c=50$, then the multiplication factor K [equation (3)] is reduced from 7 to 5 and it would not be worthwhile using the MC method in preference to a more simple EN-method ($K=4$). But it is possible that $x_c=200$ and that conditions are more favourable; only experiments can give the answer. Another problem is to what extent can we avoid the energy losses in the actual MC reactor and in the powerful accelerator. Most serious are the losses of muons in the wall, between the converter and the synthesizer. Thus Table 1 demonstrates not the actual situation, but rather the values of parameters at which mesocatalysis becomes interesting for energy production. Only by eliminating the uncertainty in all factors mentioned above will it be shown whether the MC system can be considered as an alternative method of energy production.

I thank S. S. Gerstein for discussions and for sending the reports¹⁶ which stimulated these estimates; also I. I. Gurevich, L. I. Ponomarev and Yu. M. Shabelsky for discussions and assistance, and D. I. Blokhintzev, G. N. Flerov, V. P. Dzelepov, V. N. Gribov, V. M. Lobashev, V. V. Orlov and O. I. Sumbayev for their interest and comments.

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Solid phase photoreduction of methylviologen adsorbed on cellulose

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There has been a growing interest in photochemical reduction of methylviologen (1,1'-dimethyl-4,4'-dipyridinium dichloride; MV^{2+}) as a means of solar energy conversion¹⁻⁴, because the cation radical (MV^+) can reduce protons to give hydrogen gas⁵. One problem in a photochemical conversion system is the effective separation of the reaction products, because a photochemical reaction in a homogeneous solution usually accompanies a spontaneous backward reaction which consumes the acquired energy. To overcome this problem, macro- or micro-heterogeneous reaction environments such as micelle⁶⁻⁹ or liposome¹ have been proposed. A solid phase photochemical reaction must also be useful for constructing an effective photoenergy conversion system. We describe here how MV^{2+} adsorbed on solid cellulose could be photoreduced to give MV^+ , the colour of the cellulose changing from white to deep blue. The reaction could be sensitized by tris(bipyridyl)ruthenium(II) complex ($\text{Ru}(\text{bpy})_3$) and EDTA.

Cellulose paper was dipped into a solution of 10^{-1} M MV^{2+} in methyl alcohol for 10 min and then dried under air for 15 min. This dipping and drying procedure was repeated, and the cellulose was then dried *in vacuo* at room temperature. From the UV spectra of the solution before and after dipping, the MV^{2+} adsorbed was calculated to be 382 mg per g cellulose. When this cellulose sample was irradiated with a halogen lamp (12 V, 100 W) under vacuum at 30 °C, a blue colour appeared after several minutes. The change of the transmission visible spectra of the sample accompanying the irradiation is shown in Fig. 1. The characteristic absorptions at 402 and 620 nm induced by the irradiation indicate clearly that MV^+ is formed. The shifts of the absorptions to longer wavelengths than those in water (395 and 605 nm) or in methyl alcohol (396 and 608 nm) suggest some strong adsorption of the viologen species on cellulose. The reflection

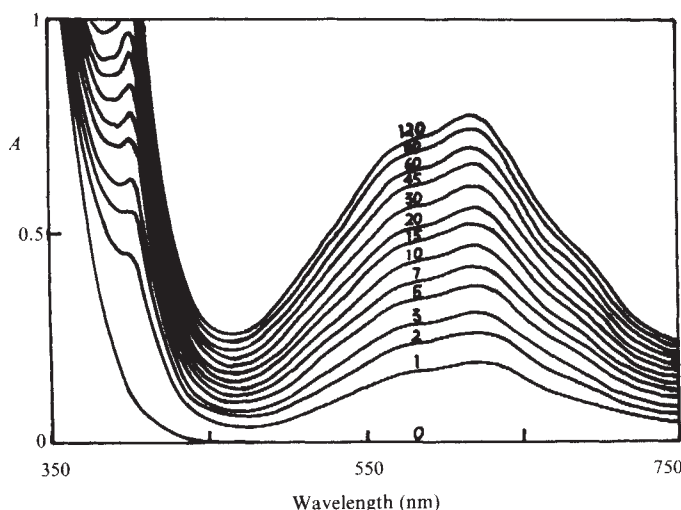


Fig. 1 Photochemical reduction of MV^{2+} adsorbed on cellulose, irradiated *in vacuo* at 30 °C. The numbers represent reaction time in min.

environment provided by cellulose favours the photoreaction, and also stabilizes the MV^+ against oxidation. Such aspects of the photochemical reactions on solid cellulose are quite different from those in micelle or liposomes. As the average distance between $Ru(bpy)_3$ and MV^{2+} or EDTA is calculated to be $<14\text{ \AA}$ on the assumption that the cellulose molecule occupies the half of the sample volume, electron transfer between the immobilized species would be possible.

Other interesting features of the solid phase photochemical reaction on cellulose are the broadening and the redshift of the absorptions of the MV^{2+} as well as of the $Ru(bpy)_3$, which leads to an utilization of wider and longer wavelengths than the corresponding solution system. The quantum yield for the production of MV^+ in the experiment *in vacuo* under 470 nm monochromatic light irradiation was estimated to be $\sim 5\%$. Natural materials containing polysaccharides were also effective as a matrix for the non-sensitized as well as sensitized photoreduction of MV^{2+} .

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Major evaporite deposition from groundwater remobilized salts

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The coastal sabkhas of the western Gulf of Sirte, Libya developed in two stages behind a barrier-ridge complex following the penultimate and the last major sea-level rises. A strong north-to-south littoral drift led to the isolation of coastal lagoons by a seaward sand ridge following the stabilization of sea level and produced isolated basins in which evaporites have been and are depositing. A sulphur isotopic study of the groundwaters and sediments described here demonstrated that the brines mostly originated in continental groundwaters supplied from the deep aquifer by surface and sub-surface flows which carry sulphate and other ions remobilized from Cretaceous sediments.

The physiography and the sedimentary pattern of the western coastal plain of the Gulf of Sirte was formed by two major transgressive events (Fig. 1). The earlier transgression of late Pleistocene age reached a level close to that of the present sea, and a barrier-island and lagoon were formed and preserved as the inner-barrier and sabkha apparently unaffected by large scale erosion during the succeeding regressive phase. The Flandrian transgression meant that the sea reached its present level $\sim 3,700\text{ yr BP}$ and it apparently spilled into the older lagoon behind the inner barrier. This outer barrier then developed to enclose a lagoon landwards, and carbonate sediments were deposited in both inner and outer lagoons. The inlets which allowed marine water to penetrate both lagoons ultimately closed and the lagoons developed into the present day inner and outer sabkha depressions. These are now the sites of deposition of modern evaporites covering more than $1,000\text{ km}^2$; their sedimentology has been described by Sherif¹.

The inflow of water into these enclosed coastal sabkhas is presently almost entirely by sub-surface flow with flooding only occurring during winter when the water table rises in response to seasonal climatic change. Subsequent evaporation of the floodwater results in precipitation of surface halite. The evaporites in the sediments of these sabkhas are dominated by gypsum and halite which are produced by evaporation and simple concentration of the groundwaters, and are arranged in a roughly concentric pattern within the sabkha depressions with gypsum at the margins and halite at the centres. Dolomite and magnesite also occur and are produced by the reaction between the lagoonal carbonate sediments and the co-existing brines.

A chemical and sulphur isotopic study of the groundwaters and sediments has enabled their origin and progress during evaporation to be defined more clearly. The concentrations of the major groundwater ions are highest in the central lagoonal basins and lowest at the two extremities and this suggests that water is supplied to the sabkhas from both landward and seaward sides. Of the three possible sources of the sabkha groundwaters—terrestrial groundwaters, seawater and meteoric water—only the first two could provide the necessary volume of salts that have been and are accumulating in these basins.

Deep terrestrial groundwaters flow into the sabkha complex by sub-surface seepage from deep fissures and by sub-surface flow from spring discharge along the landward margin of the sabkhas. The most important and the only one of these springs studied in detail^{2,3}, is that of Tawurghah which produces some $10^8\text{ m}^3\text{ yr}^{-1}$ of sulphate-rich, brackish water of $2\text{--}3\text{ g l}^{-1}$ total dissolved solids. Although the sulphate content

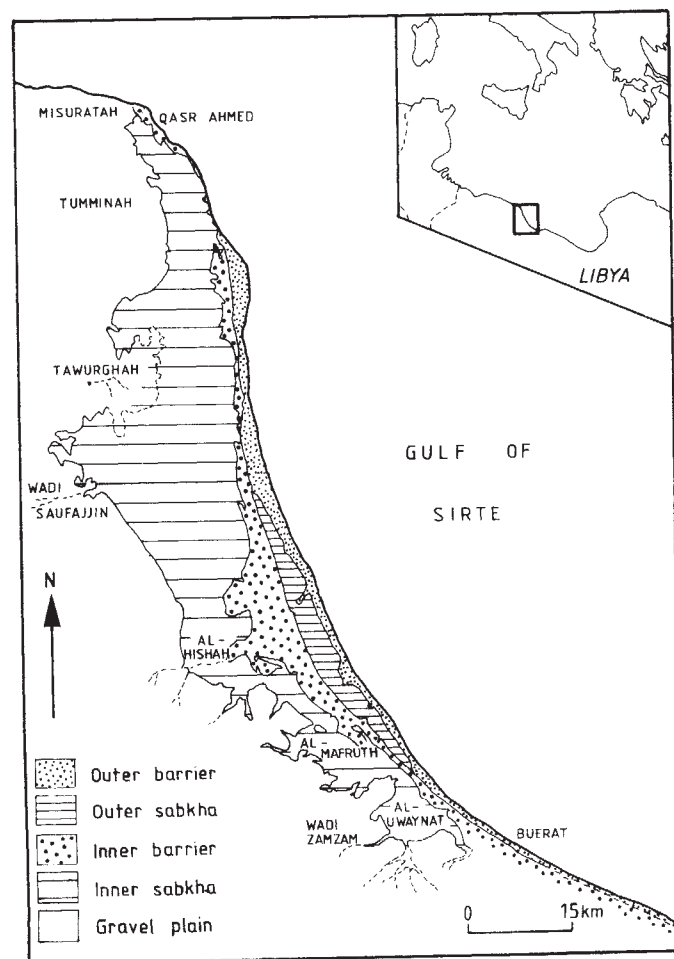


Fig. 1 The coastal plain of the western Gulf of Sirte, showing major physiographical features and sedimentary units.

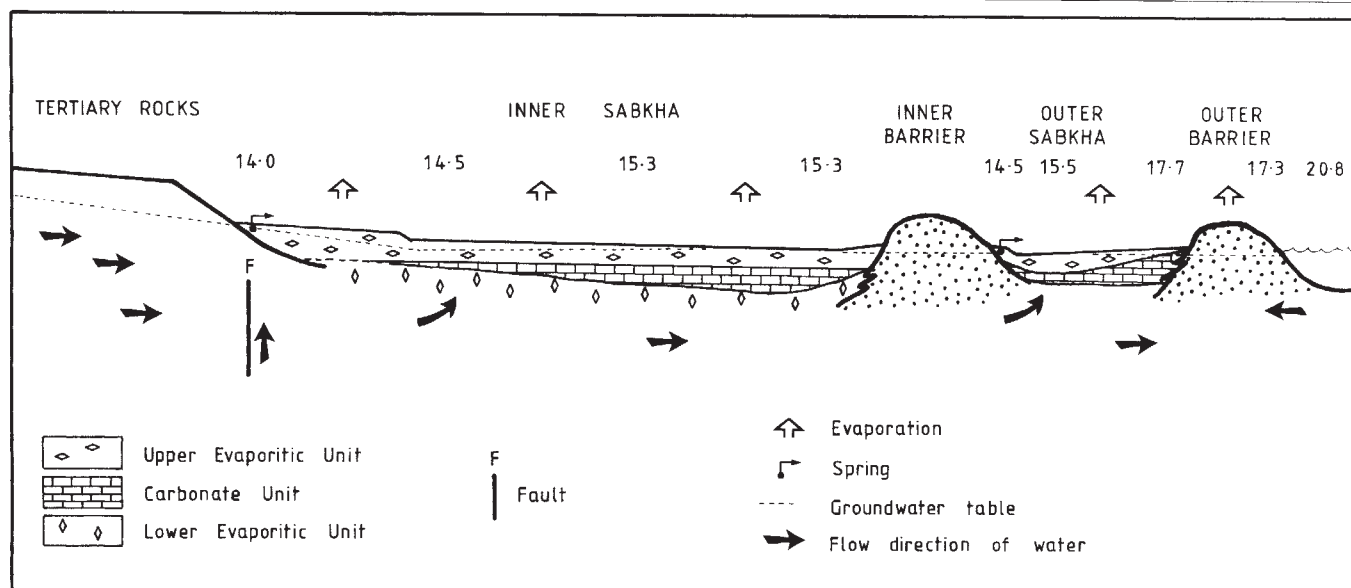


Fig. 2 The proposed hydrology of the coastal sabkha. Mean sulphur isotopic values of the groundwaters (in ‰ with respect to the Cañon Diablo meteorite standard) are superimposed above the sabkha sediment facies to which they apply.

of the various springs is very variable in the range of 0.6–5 g l⁻¹, the sulphur isotopic fractionation, $\delta^{34}\text{S}$ (with respect to the Cañon Diablo Troilite standard) is remarkably constant at $+13.9 \pm 0.5\text{‰}$.

Marine recharge of the sabkha groundwaters except during very exceptional storms by direct flooding over the seaward barrier-ridge is now very unlikely due to the height and extent of this feature. The most likely and probably only important mode of seawater encroachment is by sub-surface seepage through the barrier driven by a hydraulic head between mean sea level (MSL) and the sabkha groundwater table of ~3 m.

Eight samples of Mediterranean seawater were analysed to obtain a background sulphur isotopic value of the seawater source of sulphate. Four surface samples which might have been expected to show continental sulphate contamination, were not significantly different from four deep samples $+20.8\text{‰}$ compared with $+20.4\text{‰}$ respectively on average; nor were they significantly different from Atlantic surface water analysed at $+20.8\text{‰}$ or from the mean open ocean value of $+21.0\text{‰}$ of Rees *et al.*⁴. Salinity and sulphate content were ~10% above Atlantic levels.

Groundwater sulphur isotopic values for the inner sabkha lie in the range $+14.5$ to $+15.5\text{‰}$ and demonstrate the almost total supply of sulphate to this basin by continental waters. Only in the north, where values of $+18$ to $+20\text{‰}$ adjacent to the seaward barrier occur coincident with the maximum head between MSL and sabkha groundwater table, is there any evidence for seawater incursion. The sulphur isotopic values for the sabkha sediments, although between 1 and 2‰ heavier than the groundwaters due to the effects of water–gypsum fractionation and bacterial sulphate reduction, do not alter this conclusion. Groundwater values of the outer sabkha and the outer beach ridge grade progressively from the $+15\text{‰}$ value characteristic of the inner sabkha found at the inner barrier to $+18\text{‰}$ at the coast. One sample of coastal seawater sampled at the coast yielded an analysis of 3.8 g l⁻¹ sulphate and sulphur isotopic value of $+17.3\text{‰}$.

Considering the low isotopic values of the outer beach ridge aquifer and locally, seawater, there is good evidence to suggest that the continental water source is supplying water, and hence light sulphate, under a significant hydraulic head to both Recent and Pleistocene sand barriers and sabkhas and to the sea possibly as sub-surface springs. Only the deeper sediments with a buried algal mat have isotopic values in the region of $+20\text{‰}$. Whether these higher values are the result of

an earlier more marine phase of sabkha building before the closing of the outer barrier channels, or are due to extensive bacterial reduction and hence loss of isotopically light sulphate from the sabkha sediments as sulphides, is not yet clear.

The extensive supply of sulphate to the sabkha complex of the western Gulf of Sirte clearly originates mostly from remobilized sulphate brought to the surface and distributed into the groundwater by numerous springs of sulphate or sulphate–chloride facies water. The dominant hydrogeological feature of the area is an extensive, north–south directed fault near Tawurghah which extends vertically upwards as far as the Miocene strata. This fault and its satellite fissures provide the karstic channels through which deep artesian water from the Gharyan sediments of the Upper Cretaceous reaches the Miocene². Above this level, diffusion through fissures brings the water to the surface, both as springs and sub-surface flow, into the groundwaters of the sabkha (Fig. 2). The sediments of the Upper Cretaceous of the region are dominated by dolomite, anhydrite and shale beds; the latter providing the most likely source of the isotopically light sulphate, by dissolution and oxidation, to the waters of the deep aquifer.

These data can be compared with those from the sabkhas of the Trucial Coast where groundwater $\delta^{34}\text{S}$ values grade from $+21\text{‰}$ in the intertidal region to $+17$ to $+19\text{‰}$ at the landward edge of the Recent sedimentation^{9,10}. There, seawater remains the dominant source of sulphate. Continental sabkhas, 50–80 km inland from Abu Dhabi, exhibit $\delta^{34}\text{S}$ groundwater values between $+13$ and $+16\text{‰}$ (J.E.R. unpublished data), but the brines, though sulphate saturated, produce only small amounts of anhydrite or gypsum due to the limited supply of sulphate.

Coastal sabkhas, isolated by seaward sand barriers have been reported from Egypt⁵ and Sinai⁶. There is evidence for modern dissolution and redeposition of previous cycle evaporites in Egypt but in Sinai neither geochemistry⁷ nor isotopic analysis⁸ has demonstrated a significant continental source for the brines.

The rate of sediment production in the continental water-supplied sabkhas is dependent on the rate and salinity of water flowing from the aquifer and on the aridity of the climate to ensure a sufficiently high evaporation rate. These conditions were most likely to occur in the periods immediately following the post-glacial change to a warmer climate. Hence, according to the general variation in the regional climatology the sabkha building process may have

had a cyclic nature. An additional complication is the major variations in sea level of the past 50,000 yr, which were probably sufficiently large and rapid to prevent simple progradation of the complex, and resulted in the formation of a new sabkha barrier-ridge system as each stable point in the evolution of sea level was reached. This probably resulted in the development of a system of sabkha terraces related in height to each other by the positions of relative sea-level stability.

The present deposition rate of gypsum based on the output of the Tawurghah spring alone, is $>2 \times 10^5 \text{ ton yr}^{-1}$ and the total rate probably an order of magnitude greater, demonstrating the remarkably large amounts of sulphate that can be remobilized in a geologically short period and also showing that significant evaporite deposits associated with marine sediments need not be derived, as would generally be assumed, from the adjacent marine water, but can also be formed from water derived from terrestrial sources.

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Late Precambrian ophiolitic mélange in the eastern desert of Egypt

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Serpentinite masses in late Precambrian sediments of the eastern desert of Egypt are in a *mélange* with associated metagabbros and pillow lavas; they represent pieces of Precambrian oceanic crust. The *mélange* is of regional extent and is interpreted as a olistostrome which has been thrust northwestwards over shelf sediments and is overlain by island arc volcanics: thus the observed sequence is a structural rather than a stratigraphic one. A plate tectonic model is implied with a suture to the south-east. The interpretation of the late Precambrian (Pan-African) fold belts in Africa is controversial. It is uncertain whether, like Phanerozoic fold belts, they were formed by plate tectonic processes^{1–5}, or whether they are ensialic and unrelated to subduction of oceanic crust^{6,7} or collision of continental plates. Here we present evidence relating to this problem from the part of the Pan-African belt exposed in the eastern desert of Egypt.

According to the literature, the sequence, in simplified form, for the late Precambrian in the eastern desert is as follows⁸ (dates quoted are mostly Rb/Sr whole-rock ages, decay constants not being specified in the original publication):

- (6) Younger, post-tectonic granitoids (674–506 Myr BP)⁹.
- (5) Hammamat Group of molasse-type conglomerates, greywackes and siltstones.
- (4) Dokhan post-tectonic, calc-alkaline volcanics (~660 Myr BP)⁹.
- (3) Older, syn-tectonic, granitoids (987–830 Myr BP)⁹.
- (2) Geosynclinal metasediments (1,200–850 Myr BP)⁹ intercalated with Shadli Metavolcanic Group and intruded by serpentinites, possibly as sills⁸.
- (1) Hornblendic, psammitic and biotitic gneisses and migmatites which crop out in the Hafafit and Meatiq domes, Wadi Nugrus and east of Wadi Abu Swayel. These are thought¹⁰ to be among the oldest rocks exposed in the eastern desert. Radiometric ages range from 1,700 Myr BP (U/Pb age on zircons, probably detrital) to 592 Myr BP (K/Ar)⁹.

Mafic and ultramafic rocks, the latter mostly serpentinitized or altered to talc carbonate, are abundant in the late Precambrian of the eastern desert of Egypt and north-east Sudan as shown on a recent detailed map of part of the eastern desert¹¹ and our own work. The relationship of these serpentinites, metagabbros and epidiorites to the associated metasediments is particularly important: if they are intrusive into the metasediments they provide no support for a plate tectonic interpretation, whereas if they are ophiolites (fragments of oceanic crust) they imply a plate tectonic model. We conclude that these ultramafic and mafic rocks are not intrusives, but are fragments of oceanic crust (ophiolites) for the following reasons:

- (1) All the characteristic members of an ophiolite assemblage, including sheeted dykes, can be recognized in several localities, for example, Wadi Sodmein and along the Qena-Quseir road and also (El-Bayoumi, personal communication) in Wadi Ghadir (Fig. 1).
- (2) These ophiolitic fragments are closely associated with deep-water graphitic pelites, turbidites, cherts, olistostromes and *mélanges*.
- (3) The ultramafic bodies show no evidence of intrusion: we have found no evidence of contact metamorphism or chilling against country rocks. Instead the ultramafics form fragments mechanically incorporated in the *mélange* and in imbricate slices.
- (4) These mafic/ultramafic rocks lie in a zone which contains well documented late Precambrian ophiolites at Jabal Al Wask¹² and at Jabal Ess¹³ in Saudi Arabia and Sol Hamed¹⁴ in north-east Sudan (Fig. 1).
- (5) Analytical data so far available are compatible with an origin for these ophiolites as fragments of Proterozoic oceanic (or back-arc) lithosphere.

If it is accepted that these ultramafic and mafic assemblages are fragments of oceanic crust, it follows that they cannot be in normal superposition on the granitoid and metasedimentary gneisses which they now overlie. They must be allochthonous and the sequence given above is a structural rather than a stratigraphic sequence. All of the ophiolite contacts that we have studied in the field are tectonic. Until more detailed geochronological work is available, we regard the relative ages of the basal gneisses and migmatites, the ophiolitic *mélange* and the associated deep-water sediments as uncertain, and the accepted stratigraphic scheme as inapplicable.

Nowhere in the eastern desert of Egypt have we seen a complete, continuous and intact ophiolite sequence. Most of the ophiolitic rocks occur as fragments, isolated from other members of the ocean-floor sequence. These fragments vary in shape and angularity, and in size up to that of a mountain. They are embedded in a sedimentary matrix, usually a dark grey graphitic pelite. Ultramafic fragments are most common, with less frequent blocks of gabbro, epidiorite, pillow basalt and sediments (turbidites, chert, black limestone). Thus the ophiolitic rocks occur as fragments in a *mélange*. The field evidence suggests that this *mélange* is only 1 or 2 km thick, but extends over a vast area (at least 10,000 km²) in Wadi

Mubarak, Wadi Abu Dubbab, in the Barramiya area, in Wadi Sodmein and Wadi Atalla (Fig. 1). In the Barramiya area, ophiolitic *mélange* and normal sediments alternate as though there are several separate *mélange* sheets, but the normal sediments might be large slabs within a single *mélange*. Some of the ophiolitic fragments are very large, although the more detailed field mapping, the more the supposedly large masses are found to be composite, with sedimentary intercalations. An ultrabasic sheet at Barramiya which extends for about 30 km along strike, is preserved in synforms and overlies *mélange* and an imbricate zone with alternating thrust slices of sediment and serpentinite. The proportion of serpentinite blocks decreases downwards so that the lowest part of the sequence consists of normally bedded pelitic, turbiditic and tuffaceous sediments. This Barramiya sequence, which resembles that under the Semail ophiolite in Oman¹⁵, may suggest that the *mélange* could have underlain an ophiolite nappe. If so, this must have been entirely removed by erosion over most of the region before the deposition of the Hammamat Group, which seems unlikely. In Wadi Mubarak the *mélange* is associated with thick sequences of normal sediments (turbidites, laminated pelites) which apparently

underlie the *mélange*. Olistostromes, a few metres thick, occur within normal sediments in Wadi Mubarak and in Barramiya, but they only contain sedimentary, not ophiolitic fragments and their origin may therefore be different from that of the ophiolitic *mélange*.

The *mélange* was clearly formed and emplaced, sometimes in imbricated thrust slices, before the development of the earliest regional cleavage. Tight pre-cleavage folding is evident in some of the metasediments from changes in facing direction without corresponding change in bedding/cleavage sense; similar tight pre-cleavage folds affect the *mélange* in the Barramiya area.

The structural sections we have drawn demonstrate that the allochthonous *mélange* and the ophiolites are underlain by gneisses some of which are psammitic metasediments of shallow-water and presumably shelf origin (they contain 1,700 Myr BP detrital zircons which cannot have come from a late Proterozoic island arc), for example, Wadi Nugrus, while they are overlain by calc-alkaline volcanics, including basalts, andesites and ignimbrites, of island-arc affinity. These calc-alkaline volcanic rocks have nowhere been seen to be involved in the *mélange*. Unlike the rocks in the *mélange*, they occur in an orderly sequence, and are often relatively undeformed. They resemble, and are conventionally correlated with the Dokhan volcanics. We believe that they were erupted after the *mélange* was emplaced, but we have not seen a clear contact of volcanics unconformable on the *mélange*, and in Wadi Sodmein there seems to be no structural or metamorphic break between the *mélange* and the calc-alkaline volcanics.

There is no decisive evidence to show whether the *mélange* was an olistostrome (submarine slide deposit) or a tectonic *mélange* or both. The angular rather than lenticular shape of the fragments, lack of penetrative tectonic fabric (other than later cleavage or schistosity) in the matrix, apparently random distribution of fragments of different rock types in the matrix and small thickness of the *mélange* relative to its extent suggests that it is an olistostrome. It could not have been pushed and there is no evidence of an overlying thrust sheet under which it was dragged. In field characteristics it resembles the Monian *mélange* of Anglesey^{16,17} and the argille scagliose of the Appennines¹⁸, which are both regarded as olistostromes. However, in some localities, for example, Wadi Sodmein, tectonic deformation clearly contributed to the fragmentation of the *mélange*. The superposition of *mélange* with ocean-floor fragments on shelf sediments seems to imply extensive thrusting because it is difficult to envisage a situation where shelf sediments were at a lower bathymetric level than ocean floor. Therefore we believe that the *mélange* was formed as an olistostrome (itself presumably actuated by tectonic processes), which was subsequently thrust over shelf sediments.

Extensive tectonic transport is indicated by the presence, particularly in the gneisses underlying the *mélange*, of a regionally recumbent schistosity (folded over the Meatiq and Hafafit domes) and by a locally very intense stretching. This stretching direction, shown by the elongation of pebbles, tuff fragments, vesicles and other strain markers, trends consistently NW-SE. The direction of tectonic transport is thought to be towards the north-west, partly because of fold vergence and partly because there is no plausible source to the north-west. Because the *mélange* with its incorporated ophiolitic fragments is visibly underlain by metamorphosed shelf sediments at the Hafafit, Wadi Gemal and Meatiq areas, it apparently must have been transported at least 200 km northwestwards relative to the rocks beneath. Continued movement in the same direction is implied by parallel elongation of pebbles and fragments in the Hammamat Group.

We conclude that the ultramafic and associated rocks in the eastern desert of Egypt are ophiolites derived from ocean floor; that they are allochthonous, transported probably by gravity sliding, followed by thrusting onto shelf sediments from the present south-east, and that the supposed stratigraphic succession in the eastern desert is a structural, rather than a

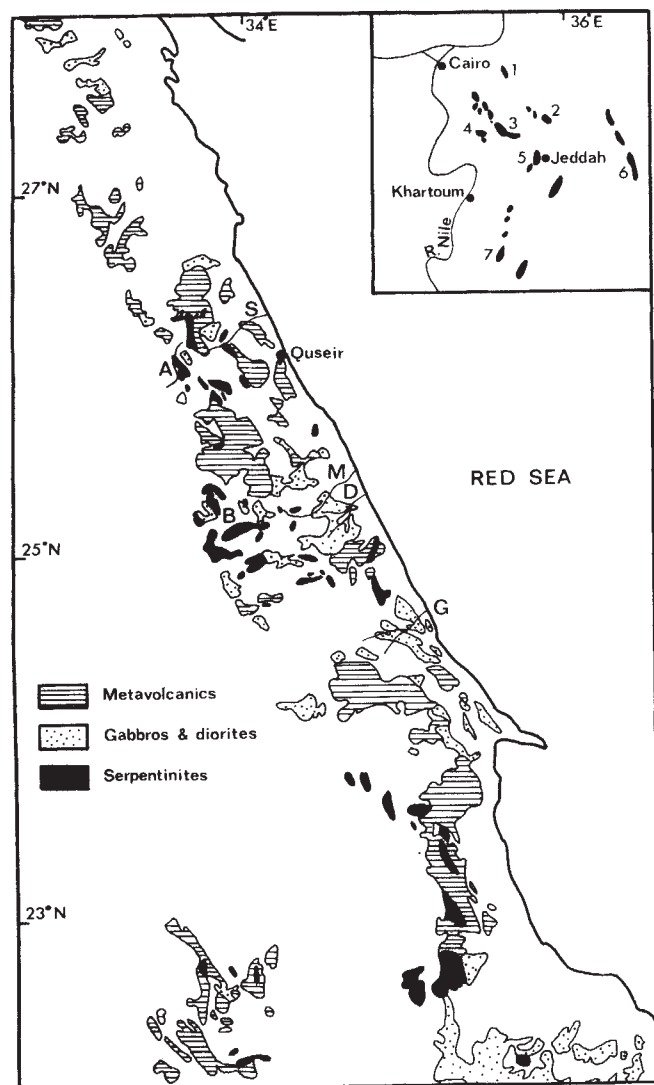


Fig. 1 Distribution of ophiolitic fragments in the eastern desert of Egypt. S, Wadi Sodmein; A, Wadi Atalla; B, Barramiya; M, Wadi Mubarak; D, Wadi Abu Dubbab; G, Wadi Gemal. Inset (reproduced with permission from I. G. Gass) shows distribution of possible ophiolites in north-east Africa and Saudi Arabia. The Red Sea has been closed. 1, Jabal Ess; 2, Jabal al Wask; 3, Quseir; 4, Barramiya; 5, Sol Hamed; 6, Bir Umq; 7, Ingessina.

stratigraphic succession. These conclusions imply a plate tectonic model, but any suture must be to the south-east of the area we have studied.

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A palaeotemperature record for the mid-Wisconsin in Vancouver Island

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Calcite speleothems are deposits of calcium carbonate (stalagmites and flowstones) found in limestone caves. They are formed by precipitation from groundwater supersaturated in Ca^{2+} and HCO_3^- . Their oxygen isotopic composition is controlled by the isotopic composition of the seepage waters from which they are deposited and by the temperature of formation. We report here the discovery of mid-Wisconsin speleothems from a cave in Vancouver Island which are significantly depleted in ^{18}O relative to modern calcite in the same cave. We interpret the variations in ^{18}O content over this period as an absolute palaeotemperature record for the area.

If calcium carbonate deposition is brought about by rapid loss of CO_2 from solution, or by evaporation of the seepage water (as may occur near the cave entrance or in a well ventilated passageway) kinetic effects will also influence the isotopic composition of the speleothem. Isotopic equilibrium deposition can be recognized in a fossil speleothem by the constancy of $\delta^{18}\text{O}_c$ and lack of correlation between $\delta^{18}\text{O}_c$ and $\delta^{13}\text{C}_c$ along given layers of contemporaneous calcite growth¹. [Expressed as

$$\delta^{18}\text{O}_x = \left(\frac{{}^{18}\text{O}/{}^{16}\text{O}(x)}{{}^{18}\text{O}/{}^{16}\text{O}(\text{PDB standard})} - 1 \right) 10^3\text{‰}$$

x = sample, c = calcite, w = seepage water, sw = seawater, p = precipitation.] Variations of $\delta^{18}\text{O}_c$ in successive growth layers of equilibrium speleothem (that is, along the growth axis of the deposit) have been interpreted in terms of temperature change, by comparison with $\delta^{18}\text{O}_c$ of speleothem calcite growing in the cave at present^{2,3} and also by analysis of D/H ratios of fluid inclusions in the speleothems^{4–6}. Time scales

were established by ^{14}C or U-series dating methods and the oxygen isotopic variations were correlated to late Pleistocene climatic events. In general, speleothems which grew during the past 100 kyr (the Wisconsin stage in North America) were found to be enriched in ^{18}O relative to modern speleothems in the same caves. For these caves, therefore, $d\delta^{18}\text{O}_c/dT < 0$, where T is the temperature inside the cave. The discovery of Wisconsin speleothems, which have a lower ^{18}O content than the modern calcite in the cave, indicates that for this area at least, $d\delta^{18}\text{O}_c/dT > 0$. The isotopic record of successive growth layers is regarded as a palaeotemperature record for the area.

The oxygen isotopic composition of cave seepage water has been shown to approximate closely to that of mean seasonal precipitation⁶. For a deep cave, well insulated from the surface, cave temperature is comparable with mean annual surface temperature. At isotopic equilibrium, the fractionation α_{c-w} between calcite and water depends only on temperature as follows⁷:

$$\Delta_{c-w} \equiv 1,000 \ln \alpha_{c-w} = 2.78 \times 10^6 T^{-2} - 2.89 \quad (1)$$

where $\alpha_{c-w} = (1 + 10^{-3} \delta^{18}\text{O}_c) / (1 + 10^{-3} \delta^{18}\text{O}_w)$. This relationship has been found to apply for ^{18}O exchange between modern calcite and cave seepage water⁸. For speleothems formed in isotopic equilibrium, variations in $\delta^{18}\text{O}_c$ along the growth axis (that is, over the period of deposition) are controlled by the following factors: (1) Effect of change in temperature of deposition on α_{c-w} ; for example, at 10°C , $d\Delta_{c-w}/dT = -0.24\text{‰ per } ^\circ\text{C}$. (2) Change in $\delta^{18}\text{O}$ of seawater; accumulation or ablation of ice on the continents causes changes in the isotopic composition of the oceans and oceanic water vapour. This causes a similar change in the isotopic composition of precipitation at the cave site. Based on changes in $\delta^{18}\text{O}$ of Foraminifera in deep sea cores, a variation of up to 1.8‰ has been suggested for a full glacial-interglacial transition⁹. (3) Effect of change in the temperature gradient between the site of evaporation and the site of precipitation on $\delta^{18}\text{O}$ of precipitation; for many oceanic and polar sites, Dansgaard¹⁰ has shown that $d\delta^{18}\text{O}/dT = +0.7\text{‰ per } ^\circ\text{C}$. CLIMAP¹¹ has shown that the tropical ocean surface temperature changed very little between full glacial and interglacial conditions and therefore the temperature change at the cave site, ΔT , is also the change in temperature gradient between evaporation and precipitation sites.

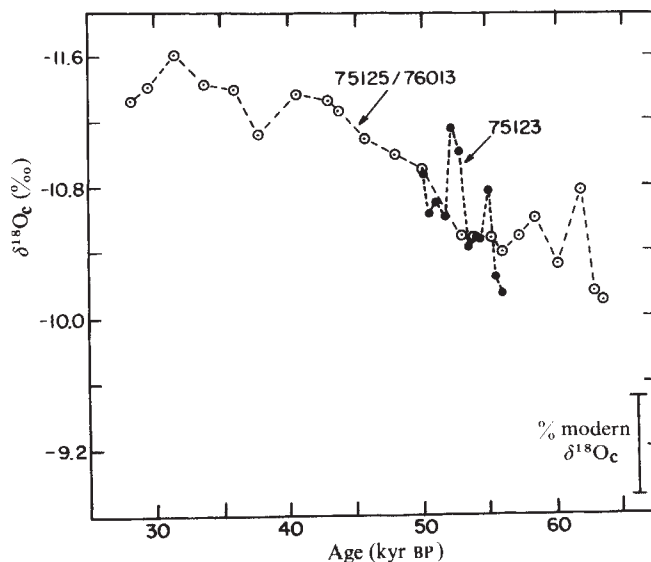


Fig. 1 Variation of $\delta^{18}\text{O}_c$ of speleothems 75125/76013 and 75123 over the dated period shown. The position of $\delta^{18}\text{O}$ of modern calcite in the same cave is shown.

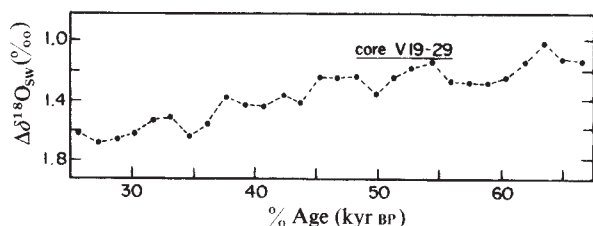


Fig. 2 Variation in $\delta^{18}\text{O}$ of seawater over mid-Wisconsin times (expressed as difference from modern values) as inferred from variations in $\delta^{18}\text{O}$ of benthonic Foraminifera⁹.

The first two effects lead to increasing $\delta^{18}\text{O}_c$ with falling temperature. The precipitation effect opposes these and causes $\delta^{18}\text{O}_c$ to decrease as temperature decreases. Variation in $\delta^{18}\text{O}_c$ of speleothem can, therefore, be summarized as:

$$\Delta\delta^{18}\text{O}_c = \frac{d\Delta_{c-w}}{dT} \cdot \Delta T + \Delta\delta^{18}\text{O}_{sw} + \frac{d\delta^{18}\text{O}_p}{dT} \cdot \Delta T \quad (2)$$

Evaluation of temperature change (ΔT) from $\delta^{18}\text{O}_c$ of ancient speleothems is hampered because the coefficient, $d\delta^{18}\text{O}_p/dT$, is commonly less than 0.7‰ per °C for precipitation at mid-continental sites and at some maritime locations^{5,12,13}. This is due to meteorological effects on $\delta^{18}\text{O}_p$, such as isotopic evolution of water vapour by precipitation from clouds at varying temperatures. Also the coefficient, 0.7, determined by Dansgaard from seasonal variations in $\delta^{18}\text{O}_p$ and temperature, may not apply to secular temperature change at a site. Lower values for speleothems which grew in North America during the Wisconsin are inferred from the enrichment in ^{18}O relative to modern deposits⁵ (variation in $\delta^{18}\text{O}_c$ is predominantly due to changes in $\delta^{18}\text{O}_{sw}$ and the effect of temperature change at the cave site on α_{c-w}). Speleothems from some maritime locations (New Zealand¹⁴, Bermuda⁵ and North-west England¹⁵) show this effect even though here $d\delta^{18}\text{O}_p/dT$ might be expected to approach the 'maritime' value of 0.7‰ per °C and so outweigh other factors.

Fossil speleothems have been collected from Cascade Cave near Port Alberni, south-central Vancouver Island. The $^{230}\text{Th}/^{234}\text{U}$ dating method has shown that many of these speleothems grew during the mid-Wisconsin interstadial 65–40 kyr BP (ref. 16). Uniformity of $\delta^{18}\text{O}_c$ along individual growth horizons of two speleothems 75123 and 76013/75125 indicates that they grew in conditions of isotopic equilibrium. Axial profiles of $\delta^{18}\text{O}_c$ are shown in Fig. 1 for the dated periods 55–50 kyr BP for 75123 and 64–28 kyr BP for 76013/75125. Analytical precision is poor for 75123 ($\pm 0.23\%$) but subsequent improvements in mass spectrometer characteristics gave a precision within $\pm 0.1\%$ for 76013/75125 analyses. Good agreement between the two profiles can be seen over the period of contemporaneous growth—a further indication of isotopic equilibrium deposition.

The profile shows that $\delta^{18}\text{O}_c$ decreases steadily by $\sim 1.3\%$ from 64 to 35 kyr BP and remains fairly constant until growth ceases at 28 kyr BP. The range of five analyses of modern calcite in the cave is also shown in Fig. 1. At all times, $\delta^{18}\text{O}_c$ (fossil) is less than $\delta^{18}\text{O}_c$ (modern). These data can be interpreted in two ways: (1) if $d\delta^{18}\text{O}_c/dT$ was negative (as found in most previous work) then mid-Wisconsin temperatures were warmer than present in this area, and became even warmer towards 28 kyr BP when growth ceased; (2) if $d\delta^{18}\text{O}_c/dT$ was positive, then mid-Wisconsin temperatures were less than present and steadily decreased towards 28 kyr BP. However, this implies that $d\delta^{18}\text{O}_p/dT$ was larger than normally observed at cave sites.

Explanation (1) conflicts with all previous work on late Pleistocene deposits in this region^{17–19} which indicates that

temperatures steadily cooled over the Olympia Interstadial (about 60–40 kyr BP) towards the maximum Fraser glaciation (~ 25 –15 kyr BP). Explanation (2) agrees well with local evidence and permits determination of temperature change over this period if $d\delta^{18}\text{O}_p/dT$ is known. We assume here a value of 0.7‰ per °C, as the setting of this cave is very similar to maritime locations at which Dansgaard and others have observed such values.

$\Delta\delta^{18}\text{O}_{sw}$ can be readily estimated from the oxygen isotopic record of benthonic Foraminifera in deep sea cores with high sedimentation rates. Shackleton has provided analytical data for the species *Uvigerina proboscidea* in core V19-29 from the Carnegie Ridge in the eastern Pacific Ocean⁹; oxygen isotope variations in this core are thought to be almost entirely due to change in oceanic isotopic composition because present ocean water temperatures at depth in this location are near 0 °C. A time scale for the core was determined by assuming constant sedimentation rate and that isotope Stage 5e, clearly seen in the core, is dated at 125 kyr BP (ref. 20). Between 64 and 28 kyr BP these data indicate an enrichment of $\sim 0.65\%$ in $\delta^{18}\text{O}_{sw}$ (Fig. 2). From equation (2), therefore, the change in $\delta^{18}\text{O}_c$ represents a decrease of ~ 4.5 °C over this period at Cascade Cave.

Combining the isotopic composition of modern Foraminifera from core V19-29 (representing modern ocean water), $\delta^{18}\text{O}_c$ of modern speleothem, and modern temperature from this cave a palaeotemperature record for the mid-Wisconsin for this area can be determined. The available core top analyses and inferences about isotopic change with respect to the isotope Stage 5e and 5 kyr BP hypsithermal isotopic minima in the core, yield an estimate of $\delta^{18}\text{O}$ of modern Foraminifera of $3.35 \pm 0.01\%$, or, 0.9‰ less than Foraminifera laid down at 64 kyr BP. $\delta^{18}\text{O}_c$ of modern calcite in the cave is $-9.2 \pm 0.3\%$. Mean annual cave temperature is estimated at 8.0 ± 0.5 °C, determined by the extrapolation to 300 m a.s.l. of mean monthly observational data from nearby meteorological stations, using the saturated adiabatic lapse rate (0.6 °C per 100 m for 5 °C).

Temperature differences between the mid-Wisconsin and present are determined from equation (2) for each of the $\delta^{18}\text{O}_c$ determinations in the speleothem, using an estimate of $\Delta\delta^{18}\text{O}_{sw}$ obtained from the V19-29 core record of Fig. 2. These temperature differences are shown in Fig. 3 to an estimated precision of ± 1 °C.

The palaeotemperature record given by these calculations seems to be quite realistic because: first, an estimate of ~ 4 °C for maximum mean annual mid-Wisconsin temperature agrees with estimates from pollen spectra in sediments in southern Vancouver Island^{17,19} and north-west Washington²¹; second, speleothem growth can only take place at or above 0 °C. These results generally comply with this requirement. Growth probably ceased as temperatures became continuously sub-zero at ~ 28 kyr BP. Use of a lower coefficient for the temperature dependence of $\delta^{18}\text{O}_p$ results in a larger temperature change, much of which is well below 0 °C, and therefore unacceptable.

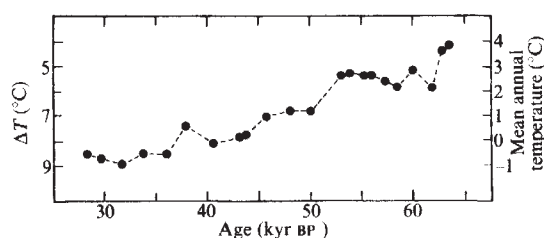


Fig. 3 Palaeotemperature curve for south-central Vancouver Island at 300 m.a.s.l. showing calculated differences between modern and ancient temperatures (ΔT), and expressed as absolute temperatures, assuming that the present cave temperature is 8 °C.

These results suggest that no strong, short-term interstadial warmings (such as may be seen in mid-continental records²²) occurred between 64 and 28 kyr BP on Vancouver Island, probably because of the damping effect of the adjacent ocean. Furthermore, there is no evidence of a pronounced cooling due to glaciation during the interval 40–30 kyr (the late Salmon Springs glaciation) as proposed by some studies of sediment sequences in Washington^{21,23}.

In conclusion, these data show a gradual cooling at this site between 65 and 30 kyr BP at an almost constant rate, comparable with the uniform rate of continental ice

accumulation suggested by Fig. 1. The local setting of this cave suggests that its temperature is responding to the average sea-surface temperatures of water masses along the northern Pacific coast and that these in turn were gradually cooling through this interval.

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Evidence for coexistence of dopamine and CCK in meso-limbic neurones

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Vanderhaeghen *et al.*¹ reported the occurrence of gastrin-like immunoreactivity in the mammalian brain. Subsequent studies have revealed that this immunoreactivity corresponded mainly to the COOH-terminal octapeptide of cholecystokinin (CCK-8), which has a COOH-terminal pentapeptide identical to gastrin^{2–7}. Also, two peptides resembling the NH- and the COOH-terminal tetrapeptide fragments of CCK-8 are present in the central nervous system (CNS)⁸. Using COOH-terminal-specific antisera raised to gastrin and/or CCK, the distribution of CCK neurones has been described with immunohistochemical techniques. Although high numbers of cells and nerve terminals are found in cortical areas, the CCK systems are also present in most other parts of the brain and spinal cord^{9–14}. In the CNS, true gastrin molecules, gastrin-17 and gastrin-34, have been located only in the neurohypophysis, hypothalamus¹⁵ and occasionally in the medulla oblongata (unpublished results). We describe here the occurrence of peptides in meso-limbic dopamine neurones in the rat brain. Evidence has also been obtained that mesencephalic dopamine neurones in the human brain contain similar peptides.

Twelve male albino rats (Sprague–Dawley, 150 g) were used. Five rats received an intraventricular injection of colchicine (60 µg in 20 µl 0.9% sodium chloride) 24 h before being killed. This mitosis inhibitor is known to arrest axoplasmic transport^{16,17} and to cause accumulation of peptides in cell bodies. Two rats received an intracerebral injection of 6-hydroxydopamine (6-OHDA) (8 µg in 2 µl 0.9% sodium chloride with 2% ascorbic acid added) into the posterior lateral hypothalamus. This treatment destroys ascending mesencephalic dopamine neurones¹⁸. The rats were perfused with ice-cold formalin as described previously¹⁹ and, after

rinsing, the brains were processed for immunohistochemistry according to Coons' indirect technique²⁰. In addition, the mesencephalon of two human post-mortem brains, fixed by immersion in formalin, were studied. Briefly, 15-µm-thick alternate sections of rat and human mesencephalon and rat forebrain were cut on a cryostat, and incubated with antiserum raised against synthetic human hexadecapeptide gastrin (antiserum 4562, specific for the C-terminus common to gastrin and CCK²¹) or against rat tyrosine hydroxylase (TH)²², a marker for dopamine and other catecholamine neurones. After rinsing, the sections were incubated with fluorescein isothiocyanate (FITC)-conjugated sheep anti-rabbit antibodies (SBL, Stockholm), rinsed, mounted and examined in a Zeiss standard fluorescence microscope. The sections from the mesencephalon of rat and human were then treated according to the following protocol. CCK-immunoreactive (fluorescent) cells in the ventral mesencephalon were photographed and then the sections were treated with acid potassium permanganate according to Tramu *et al.*²³ to elute the immunostaining. The sections were then reincubated with FITC-conjugated antibodies and examined in the fluorescence microscope. If no restaining had occurred, the sections were incubated with antiserum to rat TH and FITC-conjugated antibodies and once more examined in the fluorescence microscope and photographed. Sections incubated with antiserum 4562 preincubated with CCK-8 (50 µg per ml antiserum diluted 1:10; Peninsula Laboratories) or normal rabbit serum served as controls.

After incubation with antiserum 4562, many weakly fluorescent CCK cells were observed in the ventral mesencephalon of colchicine-treated rats. The immunoreactive cells were observed mainly in the ventral tegmental area, but cells were also seen in the zona compacta, particularly at a rostral level and in its medial part. At the mid and caudal levels a small group of fluorescent cells were consistently observed in the pars lateralis of the substantia nigra. At the caudal level the cells were mostly restricted to the midline area overlying the interpeduncular nucleus. No immunoreactive cells were seen in the pars reticulata. Alternate sections incubated with TH antiserum revealed a close overlap between TH- and CCK-immunoreactive cells, although the former were much more numerous and were found also in the zona compacta and pars reticulata, in agreement with the original description of the distribution of dopamine cells of the A9 and A10 groups by Dahlström and Fuxe²⁴. The elution and restaining experiments revealed that many of the CCK-immunoreactive cells also contain TH (Fig. 1a, b), although

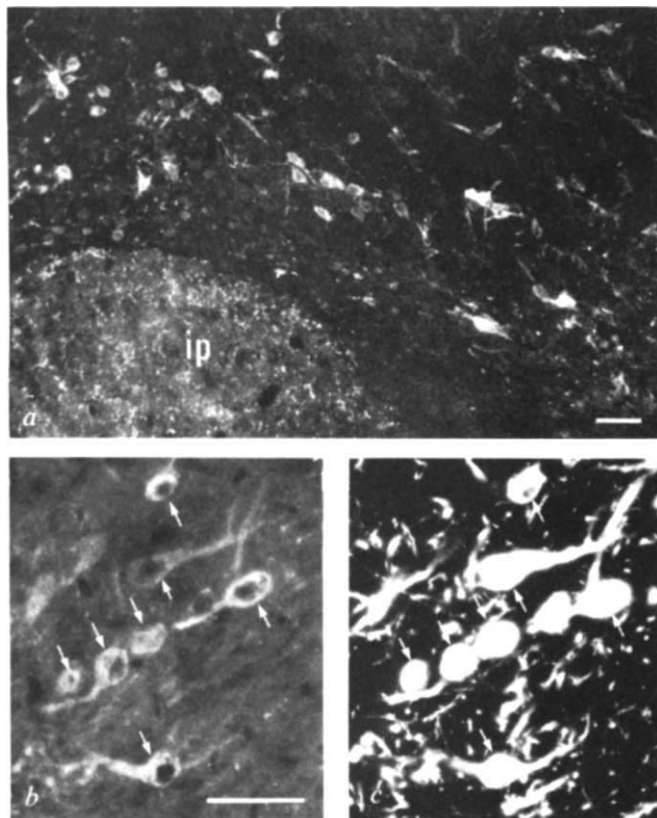


Fig. 1 *a-c*, Immunofluorescence micrographs of the ventral tegmental area of colchicine-treated rat after incubation with antisera to gastrin/CCK (*a, b*) and tyrosine hydroxylase (*c*). *b* and *c* show the same section, which, after photography (*b*) and removal of the gastrin/CCK antiserum according to Tramu *et al.*²³, has been reincubated with TH antiserum (*c*). Several fluorescent (CCK immunoreactive) cells are seen in the region overlying the interpeduncular nucleus (ip) (*a*). Note also numerous CCK-immunoreactive nerve endings in the ip (*a*). Restaining experiments show identity between CCK-immunoreactive (arrows in *b*) and dopamine (TH-immunoreactive) (arrows in *c*) containing cells. Scale bars, 50 μ m.

identity could not be established in all cases. Thus, some CCK-immunoreactive cells were TH negative and vice versa.

Numerous cells containing both CCK and TH-like immunoreactivity were observed in the human mesencephalon, although, again, a large number of cells only contained the enzyme.

In the frontal brain dense networks of CCK-immunoreactive nerve fibres were observed in, among other places, the caudal, dorsomedial parts of the nucleus accumbens, in the medial parts of the tuberculum olfactorium, in the bed nucleus of the stria terminalis (caudal and dorsal parts) and in the central amygdaloid nucleus. In the alternate sections, the TH-immunoreactive fibres were present in all these areas but in higher concentrations and more widely spread within these nuclei. There were, of course, also both CCK- and TH-immunoreactive nerve fibres in many more areas of the brain stem and telencephalon but without the obvious overlap observed in the areas described above. We will not deal with such areas here but refer to previously published immunohistochemical papers⁹⁻¹⁴.

After injection of 6-OHDA into the lateral hypothalamus, most CCK- and TH-immunoreactive fibres disappeared in the nucleus accumbens, tuberculum olfactorium and the bed nucleus of stria terminalis. After incubation with the control sera, none of the fluorescent structures described above were

observed. This was also the case when the respective control serum was included in the restaining experiments.

The present findings are strong evidence that some dopamine neurones in rat and man also contain CCK peptides. Preliminary results in our laboratories indicate that, as with other brain regions, the CCK-like immunoreactivity in the dopamine neurones corresponds to CCK-8.

In the rat, only a subpopulation of the mesencephalic dopamine neurones contained CCK-like peptides. Their distribution corresponds well to the so-called A10 group located in the ventral tegmental area² but some neurones were also seen in the pars compacta and pars lateralis. The A10 dopamine cells are known to project mainly to certain limbic areas^{25,26} and exactly in these areas we observed, on adjacent sections, overlapping dense networks of CCK- and TH-immunoreactive nerve endings. The fact that both CCK- and TH-immunoreactive fibres in these areas disappear after 6-OHDA treatment further strongly supports the coexistence hypothesis.

The present results offer a further example of coexistence of small regulatory peptides and a biogenic amine in neurones²⁷. Previously, for example, somatostatin has been found in noradrenaline neurones²⁸ and substance P in 5-hydroxytryptamine neurones^{29,30}. Such situations were early observed in endocrine cells, which according to Pearse's terminology belong to the APUD (amine precursor uptake and decarboxylation) system³¹.

Evidence is accumulating that CCK-8 and/or CCK-4 may act as a neurotransmitter in the brain³². In addition to the morphological evidence cited above, it has recently been shown that CCK peptides can be released from synaptosome-enriched fractions of rat cerebral cortex by potassium^{32,33}. Furthermore, in the hippocampus, application of CCK-8 and CCK-4 causes excitation, possibly through a direct action on pyramidal cell somata³⁴. Thus, a population of meso-limbic dopamine neurones contains, in addition to dopamine, a second peptidergic putative transmitter.

The occurrence of an additional peptide transmitter and/or modulator in some dopamine neurones is interesting in view of the hypothesis of an involvement of these neurones in various nervous disorders such as Parkinson's disease^{35,36} and schizophrenia³⁷.

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Diurnal cycles in serotonin acetyltransferase activity and cyclic GMP content of cultured chick pineal glands

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Levels of serotonin *N*-acetyltransferase (NAT: acetyl CoA:arylamine *N*-acetyltransferase; EC 2.1.1.5.) activity in the chick pineal gland exhibit a marked diurnal variation in birds kept under a diurnal cycle of illumination¹⁻⁴. Activity begins to rise rapidly at the start of the dark phase of the cycle and reaches maximum levels at mid-dark phase about 25-fold greater than the minimum basal level at mid-light phase. Thereafter, the level of activity declines to the basal level about the start of the light phase. This diurnal cycle in chick pineal NAT activity found *in vivo* has recently been reproduced *in vitro* with intact glands incubated in organ culture⁴⁻⁷. The mechanism of the 'biological clock' which regulates these variations in level of chick pineal NAT activity is unknown. However, I now report that chick pineal glands cultured under a diurnal cycle of illumination exhibit a diurnal cycle in content of cyclic GMP which roughly parallels the cycle in NAT activity. In contrast, there was no correlation between variations in pineal content of cyclic AMP and in level of NAT activity.

A very marked stimulation of the 'nocturnal' (dark phase)^{4,8} increase of NAT activity occurs in chick pineal glands cultured under a diurnal cycle of illumination on treatment with the combination of theophylline with compound Ro20.1724 (4[3-butoxy-4-methoxybenzyl]-2-imidazolidinone), which are inhibitors of cyclic nucleotide phosphodiesterase activities⁹. This combination of inhibitors also induces a marked increase of NAT activity in glands cultured under continuous illumination⁸, and almost totally abolishes the inhibition of increase of NAT activity by light (unpublished data). These observations are circumstantial evidence of a possible role of cyclic nucleotides in the 'biological clock' of the chick pineal gland. Therefore, I have followed the course of variations in pineal contents of cyclic AMP and cyclic GMP in glands incubated in organ culture under a diurnal cycle of illumination.

White Leghorn male chicks were maintained under a diurnal cycle of illumination with a photoperiod of 14 h between 0100 and 1500 h at a light intensity of ~1,000 lx at the level of the birds, and a dark period of 10 h (Figs 1 and 2). Pineal glands were removed from 1-week-old chicks (7-9 days after hatching)⁴ between 1030 and 1100 h and cultured under

the same standard diurnal cycle of illumination, with light at an intensity of ~1,000 lx during the photoperiod.

The pineal cyclic GMP content of glands cultured in our standard conditions⁸ and the standard diurnal cycle of illumination appeared to vary in a diurnal manner (Fig. 1, curve A). It rose about fourfold during the dark phase of the cycle of illumination to a maximum at mid-dark phase, decreased rapidly in the early photoperiod to a minimum, and rose again during the dark phase of a second cycle of illumination. Decrease of pineal cyclic GMP content in the early photoperiod was not dependent on exposure to visible light, for a corresponding decrease was seen during the same interval of (clock) time in a smaller series of experiments with glands cultured in continuous darkness (curve B).

The diurnal cycle in pineal cyclic GMP content under a diurnal cycle of illumination roughly paralleled the cycle in level of pineal NAT activity (Fig. 1, curve C).

A marked difference was found between the variations in pineal cyclic GMP content and NAT activity when glands were cultured under the diurnal cycle of illumination in the presence of theophylline plus compound Ro20.1724 (Fig. 2). The cyclic GMP content (Fig. 2a) increased without apparent lag during the final hours of the photoperiod, but reached a

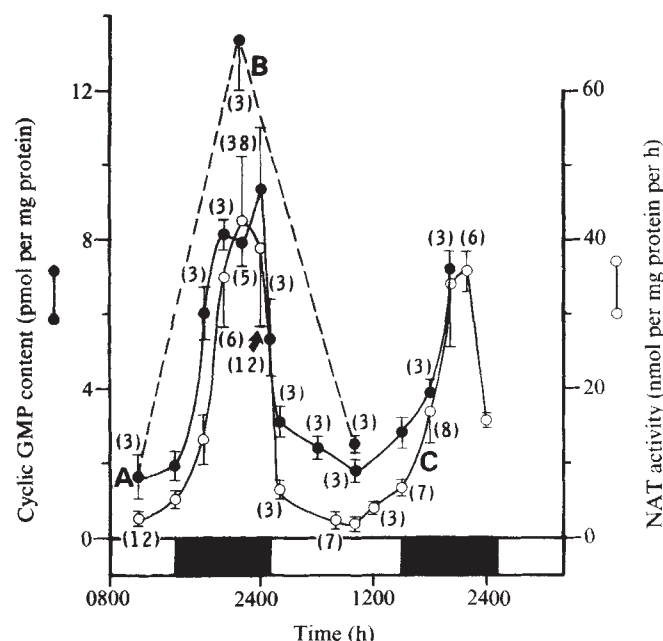


Fig. 1 Diurnal variations in chick pineal NAT activity and cyclic GMP content in culture. Groups of three pineal glands each were distributed into mini-organ cultures with 0.24 ml of medium 199 with Earle's salts¹² supplemented with antibiotics and 20% heat-inactivated fetal calf serum (Gibco)^{11,13}. The cultures were incubated under 5% CO₂ in oxygen standard to the times indicated under the diurnal light cycle (bar diagram) at an intensity of ~1,000 lx, at 38 °C in a constant-environment room with forced air circulation. All glands were recovered as rapidly as possible, rinsed with saline, blotted and frozen by immersion in liquid N₂ under dim red light (~40 lx), regardless of lighting conditions at the end of the incubation period⁴. Cyclic GMP content (curve A) was determined on pools of six pineal glands by the radioimmunoassay method of Frandsen and Krishna¹⁴, using commercial RIA kits (NEN). No correction was made for levels of 'apparent cyclic GMP' (<0.05 pmol per mg protein) due to cross-reaction with cyclic AMP. Additional values for control glands incubated in constant darkness are given in curve B. NAT specific activity (curve C) was determined on pools of three glands with tryptamine as amine substrate as previously described⁴. Protein was determined by the method of Lowry *et al.*¹⁵, with bovine serum albumin as standard. Numbers of independent determinations were either four or the value in parentheses.

peak value at mid-dark phase which was not significantly greater ($t = 1.803$, d.f. = 7, $P > 0.1$) than that found with control glands (curves A and B). The combination of cyclic nucleotide phosphodiesterase inhibitors markedly inhibited the subsequent decrease in pineal cyclic AMP content in the photoperiod.

When cultures were transferred to continuous darkness at mid-dark phase of the first diurnal cycle of illumination (curve C) the inhibition of subsequent decrease of cyclic GMP content was even more markedly inhibited than in glands exposed to light (curve B).

As previously reported⁸, the combination of theophylline with compound Ro20.1724 markedly stimulated increase of NAT activity in the light without appreciable lag (Fig. 2a, curve B from 1100 to 1500 h on day 1 of culture). The increase of NAT activity continued in the dark to a peak level at mid-dark phase which was about four times greater than that found with control glands (curve A). The level of NAT activity decreased in the following photoperiod, but at a slower rate of net decrease than in the control glands.

This decrease of NAT activity could not be attributed to toxic effects of the drug combination. NAT activity rose again during the dark phase of a second cycle of illumination to levels at least equal to the peak level developed during the first cycle of illumination in culture (curve B). Moreover, the rate of decrease of NAT activity seen with cultures containing the inhibitors during the photoperiod (curve B) was markedly further reduced when the cultures were transferred to constant darkness shortly before start of the photoperiod (curve C).

There was no correlation between content of cyclic AMP and level of NAT activity in control pineal glands cultured under the diurnal cycle of illumination (Table 1). The cyclic AMP content after 4 h of culture was roughly half the initial content *in vivo* at the time of killing (1100 h) and did not change significantly during at least 15 h of further incubation. In the presence of 1 mM each of theophylline and compound Ro20.1724, the pineal cyclic AMP content increased roughly 10-fold to 223 ± 58 pmol per mg protein ($n = 3$) at 2200 h.

Assays of the pineal cyclic AMP content *in vivo* (Table 1) also gave no indication of any correlation between pineal cyclic AMP control and level of NAT activity.

I was unable to obtain reliable estimates of the cyclic GMP content of the chick pineal gland *in vivo* during the dark phase of the cycle of illumination. Observed values tended to be higher than during the photoperiod but were extremely variable. For example, nine values determined with glands removed 3 h after start of the dark phase ranged from 1.1 to 6.5 pmol per mg protein, with a modal value of 2.6. Part of this variability could possibly be attributed to differences in degree of activity exhibited by the birds, which may have reflected disturbance and attendant stress. (Noradrenaline depresses the level of NAT activity in cultured chick pineal glands^{10,11}.) I assume that the variability also reflects variations in length of interval between killing of the bird and freezing of the gland (average 45 s in the dark, 5 s for removal of glands from culture), and hence of duration of exposure to dim red light (legend to Fig. 1) and of postmortem metabolism.

The mechanisms of action whereby theophylline and compound Ro20.1724 elicit marked increases of NAT activity in the chick pineal gland are not known. The effects of these agents on cultured chick pineal glands have been examined for two related reasons^{4,5,8,16}. First, both are known to be effective inhibitors of cyclic nucleotide phosphodiesterase activity⁹. Second, regulation of the diurnal cycle in level of NAT activity in the rat pineal gland seems to be mediated through variations in pineal content of cyclic AMP^{17,18}.

However, the mechanism regulating the diurnal cycle of NAT activity in the chick pineal gland differs in several important respects from that controlling the corresponding cycle in the rat pineal gland^{8,19}. Therefore, increase of pineal cyclic AMP content and/or level of NAT activity in chick

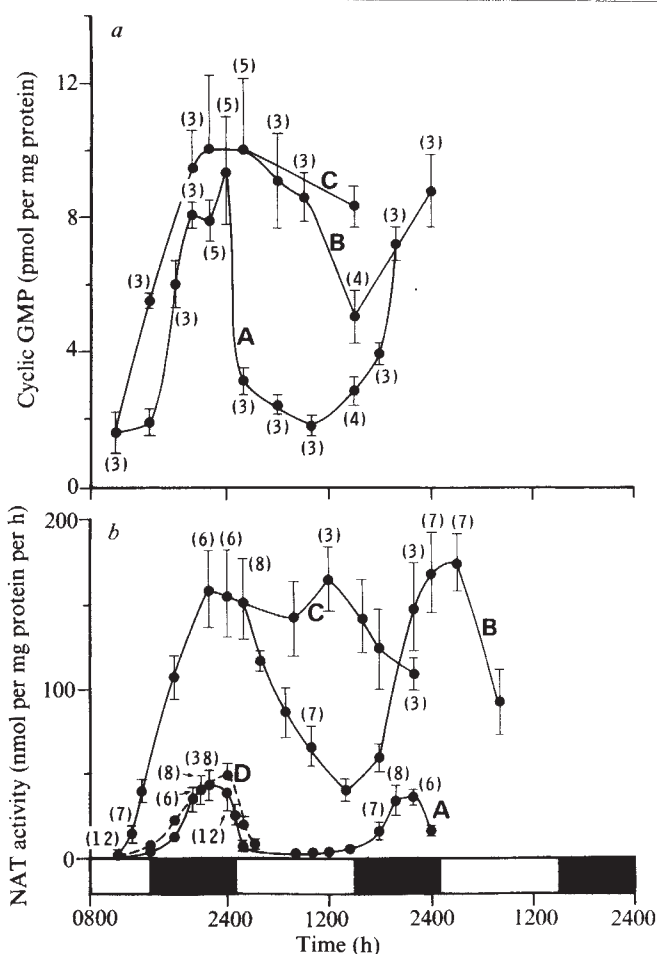


Fig. 2 Effects on the diurnal cycles in cyclic GMP content and NAT activity in culture of theophylline and Ro20.1724. Pineal glands from birds kept under the standard diurnal light cycle (bar diagram) were cultured with control medium under the standard light cycle (curve A), with 1 mM theophylline plus 1 mM Ro20.1724 under the same light cycle (curve B), and under the same light cycle to 2400 h on the first day of culture followed by continuous darkness (curve C). Levels of NAT activity in control cultures incubated in constant darkness are also given (curve D). Other details were as in the legend to Fig. 1. Levels of 'apparent cyclic AMP' due to cross-reaction of the cyclic GMP-specific antiserum with cyclic AMP were less than the s.d. of the cyclic GMP contents assayed and therefore were not corrected for. Numbers of independent determinations were either four or the value in parentheses.

pineal glands exposed to one or both of these agents is not adequate evidence that level of enzyme activity is regulated by level of cyclic GMP content.

Similarly, dibutyryl-cyclic AMP has been reported to act as an inhibitor of rat pineal cyclic phosphodiesterase activity²⁰. Therefore it is imprudent to conclude that the small and variable increases of NAT activity elicited by addition of this cyclic AMP analogue to cultured chick pineal glands seen by ourselves¹¹ and others^{5,16} strongly implicate cyclic AMP, rather than cyclic GMP, in regulation of the diurnal cycle of NAT activity in the chick pineal gland.

Indeed, the results obtained in this study (Table 1) are not readily compatible with the hypothesis that the normal diurnal cycle of NAT activity in the chick pineal gland is regulated by variations in cyclic AMP content.

In contrast, the parallel diurnal variations in both cyclic GMP content and NAT activity (Fig. 1) are compatible with the hypothesis that the variations in cyclic GMP content play an important part in regulating the normal diurnal cycle of NAT activity in the chick pineal gland.

Moreover, I assume that any effect of increased pineal cyclic GMP content on NAT activity is mediated through an increase of cyclic GMP-dependent protein kinase activity. On the other hand, reversal of that effect after a subsequent decrease of cyclic GMP content would require both a decrease of the kinase activity and the action of a protein phosphatase. Thus effects of a premature, but sustained, increase of pineal cyclic GMP content elicited by the combination of theophylline plus compound Ro20.1724 (Fig. 2a, curves A and C) could be greatly amplified in the very marked stimulation of increase of NAT activity (Fig. 2b, curves A and C).

Table 1 Variations in chick pineal cyclic AMP content

Time (h)	pmol cyclic AMP per mg protein	
	in culture	in vivo
1100	38.0 ± 4.0 (7)	38.0 ± 4.0 (7)
1500	15.6 ± 4.9 (6)	30.7 ± 6.8 (5)
1630	20.6 ± 5.3 (3)	—
1700	22.1 ± 4.3 (8)	—
1800	—	26.7 ± 8.5 (3)
2000	25.6 (2)	21.2 (2)
2200	22.5 ± 4.3 (12)	31.4 ± 5.8 (5)
2400	22.8 ± 5.3 (5)	—
0300	—	28.9 (2)
0800	19.3 ± 4.6 (4)	—

White Leghorn chicks were kept under the standard light cycle and cyclic AMP contents *in vivo* were determined for birds killed at the times indicated. Dissections for birds in the dark were by dim red light⁴. The radioimmunoassay method for Frandsen and Krishna¹⁴ was used for determination of cyclic AMP, using commercial kits. No correction for cross-reaction with cyclic GMP was necessary. All other methods were as given in the legend to Fig. 1. Numbers of independent determinations are given in parentheses.

Nevertheless, the NAT activity elicited by the drug combination declines rapidly in the normal photoperiod (Fig. 2b, curve B), whereas decrease of pineal cyclic GMP content is markedly inhibited (Fig. 2a, curve B). Further, supplements of exogenous dibutyl-cyclic GMP have been recently reported to depress the level of NAT activity in cultured chick pineal glands¹⁶.

Therefore it seems that the diurnal cycle of NAT activity in the chick pineal gland is probably not determined solely by changes in pineal cyclic GMP content. Indeed, it is possible that the parallel variations of pineal cyclic GMP content and of NAT activity are the fortuitous coincidence of two unrelated cycles controlled by a single 'biological clock'.

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Prostacyclin production by cultured smooth muscle cells from atherosclerotic rabbit aorta

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Prostacyclin (PGI₂) synthesis seems to be one of the major physiological mechanisms involved in regulating platelet and vessel wall interactions¹. PGI₂ is produced in large amounts by vascular endothelial cells, and vascular smooth muscle cells (SMC) also produce significant quantities². The capacity of SMC to produce PGI₂, especially after endothelial injury, seems to be of importance. It is probably this type of situation that is involved in the atherosclerotic process³: experimental atherosclerosis in rabbits has been associated with a severe decrease in PGI₂ synthesis by arteries⁴. Lipid peroxide accumulation within the arterial wall or in the plasma may also be involved in this process⁵. Using arterial SMC in culture, we demonstrate here that, in comparison with healthy cultured cells, cells originating from atherosclerotic aorta have a decreased capacity to produce PGI₂. The results were obtained using biological and radiochemical techniques and were confirmed by GC-MS. They suggest a potential role for PGI₂ in inhibiting the atherosclerotic process.

Experiments were carried out with 1.5-yr-old rabbits. Smooth muscle cells derived from explants of the medial layer from thoracic aorta in both normal and atherosclerotic animals (cholesterol, 500 mg per day for 6 months, then maintained for 6 months on a normal diet) were grown at 37 °C in 75-cm³ plastic flasks in an atmosphere of 5% CO₂ in air essentially as described by Ross⁶. Cells were used after 5–10 trypsinizations.

At confluency cells were washed twice with serum-free culture medium, collected by scraping with a rubber policeman and suspended in Tris (0.05 M), NaCl (0.15 M) pH 7.4 buffer. Aliquots of 0.2 ml were used for protein concentration determinations according to the method of Lowry. Bovine serum albumin was used as standard. Cell counts were performed with a haemocytometer after trypsinization of cells from flasks at the same passage.

In previous studies, aliquots of cell suspensions were incubated in Tris-NaCl buffer at 22 °C. SMC incubated for 10 min released an inhibitor of ADP induced aggregation. Aliquots (100 µl) of supernatants from incubations of 10⁶ healthy and atherosclerotic cells induced an inhibition of 44% and 13%, respectively. In these experiments inhibitory activity was not detected in buffer incubated without cells or in buffer alone incubated directly with the cell layer attached to the flask. No inhibitory activity was detected in supernatants from cells pretreated with aspirin (5 × 10⁻² M) for 10 min, after heating (15 min, 37 °C) or acidification.

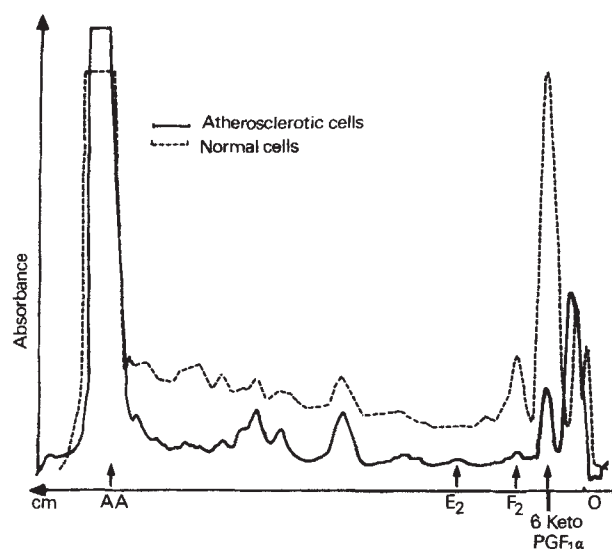


Fig. 1 Scan of autoradiogram observed after 3 days of exposure. Absorbance was measured at 550 nm. Aliquots of 20 μ l ethyl acetate dissolved extracts were chromatographed on silica gel G plates previously activated for 30 min at 110°C and developed by ascending chromatography in the organic layer of ethyl acetate, isooctane, acetic acid, water (90:50:20:100 v/v). Arrows indicate the position of unlabelled standards visualized by iodine vapours. F₂, PGF_{2 α} ; E₂, PGE₂; AA, arachidonic acid.

Suspensions of cells from several normal or atherosclerotic strains were incubated with ¹⁴C-labelled arachidonic acid (AA). After incubation, the mixture was acidified to pH 2 and extracted with ethyl acetate. The organic phase was evaporated to dryness under nitrogen. Dried extracts were redissolved in 0.1 ml ethyl acetate and subjected to TLC. As shown in Fig. 1, a major area of radioactivity was co-chromatographed with 6 keto-PGF_{1 α} standard (the stable breakdown product of PGI₂). In other experiments, aspirin (5×10^{-2} M) was added 10 min before incubation: production of 6 keto-PGF_{1 α} was markedly reduced (Table 1).

Different cultures exhibited different rates of conversion of AA to its metabolites: limit values were 1.27 to 2.60% for healthy cells and 0.32 to 0.85% for atherosclerotic cells. The number of passages did not greatly influence PGI₂ synthesis between 5–10 passages, as per cent transformations ranged from 1.72 to 1.98 for L735 healthy strain cells and from 0.39 to 0.34 for L728 atherosclerotic strain cells.

Disparities occurred between different cultures from the same tissue type. This phenomenon has already been described by other authors⁷. However, it seemed obvious that in comparison with normal cells, atherosclerotic cells exhibited a strong decrease in their capacities to synthesize PGI₂ from exogenous AA (per cent transformation 0.60 ± 0.28 and 2.00 ± 0.48 , respectively; $P \leq 0.01$).

We defined this decrease further with GC-MS determinations. Prostacyclin formed after a 30-min incubation of cells with 10 μ g AA was measured as 6 keto-PGF_{1 α} , as previously described⁸. Briefly, prostaglandin F_{2 β} (PGF_{2 β}) was added as internal standard before extraction with ethyl acetate at pH 3. The organic phases were pooled and evaporated to dryness under nitrogen and the biological extract subjected to silicic acid chromatography. The collected fractions were evaporated and derivated to methyl esters, methoximes and trimethylsilyl ethers. Methyl esters were prepared by treatment with anhydrous ethereal diazomethane (1.0 ml) for 1 h at room temperature. After esterification the excess reagents were evaporated at 25°C with nitrogen. Methyl ester of 6-keto-PGF_{1 α} was converted into methoxime by heating (60°C, 1 h) the residue left after esterification with a saturated solution

(0.1 ml) of *O*-methylhydroxylamine chloride in anhydrous pyridine. Pyridine was evaporated with nitrogen. PGF_{2 β} and 6 keto-PGF_{1 α} ester oximes were converted into trimethylsilyl ethers by heating at 60°C for 1 h with *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) (95/5, v/v).

We monitored the metabolic profiles of AA in cell preparations with glass capillary-gas chromatography and multiple ion detection (GC-MS LKB 2091). Figure 2 shows the transformation of AA incubated with normal and atherosclerotic cultured SMC. Measurements performed on different cell homogenates from L7805 healthy and L7804 atherosclerotic strains at different passages showed that 271–1,471 ng per mg protein (mean 918 ± 574) and 185–476 ng per mg protein (mean 285 ± 165) of 6 keto-PGF_{1 α} was recovered from the healthy strain and the atherosclerotic strain, respectively ($P \leq 0.05$).

Three results demonstrate that healthy vascular smooth muscle cells in culture can synthesize PGI₂ in relatively large amounts. This finding is in agreement with values for human

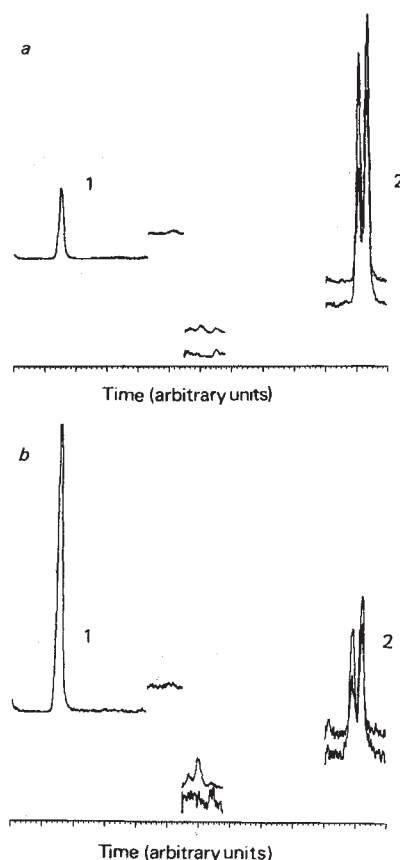


Fig. 2 Multiple ion monitoring of characteristic ions in the mass spectra of: (1) methyl ester trimethylsilyl ether PGF_{2 β} , $m/e = 423$: $M^+ - (Si - (CH_3)_3 - OH + C_5H_{11}) \times 25$; (2) methyl ester oxime trimethylsilyl ether 6 keto-PGF_{1 α} , $m/e = 378$, $M^+ - (Si - (CH_3)_3 - OH + C_5H_{11}) \times 200$. The two peaks recorded at m/e 378 and m/e 418 are the *syn* and *anti* isomers of the methoxime derivative of 6 keto-PGF_{1 α} . The recording of the two extracts, a, normal strain, b, atherosclerotic strain, use the same amplification and scale factors, therefore the area ratios, internal standard/measured product, demonstrate the difference between the two strains. Calibration curves for multiple ion detection of PGF_{2 β} and 6-keto-PGF_{1 α} allowed quantification. GC was performed with a home-made wall coated open tubular column (length, 20 m, i.d. 0.30 mm; theoretical plates $3,500 m^{-1}$, stationary phase: OV₁). Oven temperature ranged from 200°C to 265°C (2°C min⁻¹).

Table 1 Conversion of arachidonic acid to 6-keto-PGF_{1α} and influence of aspirin treatment

Tissue origin	Aspirin treatment	No. of experiments	6-keto-PGF _{1α} (mean ± s.d.)
Healthy media	No	12	2.30 ± 0.21
L7805	Yes	6	0.46 ± 0.08
Atherosclerotic media	No	10	0.86 ± 0.17
L7804	Yes	5	0.20 ± 0.05

Results were expressed as the per cent transformation of 10 nM AA by 500 µg protein of SMC in a total volume of 0.5 ml within the 20 min direct incubation period at room temperature, or after 10 min preincubation with 5×10^{-2} M aspirin.

vascular smooth muscle cells reported by others and is similar to those obtained with fresh arterial intimal cell suspensions².

Atherosclerotic cells in culture synthesize PGI₂ at a lower rate than do normal cells. Further experiments are required to explain these differences. Several mechanisms may be involved in this process. A decrease of spontaneous generation of prostacyclin by atherosclerotic arterial rings has previously been described⁴. This phenomenon has been explained by an increase of lipid peroxidation which inhibits prostacyclin synthesis⁵. We have shown that, *in vitro*, in the same culture conditions (particularly the same lipid concentration), a decrease of prostacyclin synthetase activity occurs in cells originated from atherosclerotic aortas. Additional results (data

not shown) demonstrate that cyclooxygenase may be disturbed in the latter case but do not seem to indicate that another pathway for prostaglandin formation occurs. An increased formation of PGE₂ or PGD₂ as reported in atherosclerotic rabbit aortic rings⁵, was not observed. The identification of lipoxygenase activity in vascular tissue^{10,11} may be important in explaining the modifications observed, as the AA hydroperoxides may modulate PGI₂ production in vascular tissue by inhibiting prostacyclin synthetase⁵.

Our findings suggest that the reduced capacity of atherosclerotic smooth muscle cells to produce PGI₂ may be significantly involved in the course of the atherosclerotic process.

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Pressure inhibits thermal killing of Chinese hamster ovary fibroblasts

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The increasing application of hyperthermia to cancer therapy^{1–5} has resulted in intensive study of the cellular effects of small temperature elevations (from about 37 to 43 °C). The critical heat target(s) have not been identified, however, findings such as the alterations in membrane morphology, inhibition of potassium transport⁶ and increased permeability to a variety of substances^{7,8} implicate the plasma membrane⁹. Also, there are striking similarities between the response of cells in tissue culture to heat and various membrane fluidizing agents, including anaesthetics and alcohols^{10–14}. An initial exposure of cells to non-lethal doses of heat will trigger development of subsequent resistance to both heat and ethanol, as well as to adriamycin. Similarly, an initial exposure to ethanol will result in subsequent resistance to all three of these agents^{10–12}. A range of anaesthetics and alcohols also act synergistically with heat in cell killing^{12–14}. As the effects of ethanol and many other anaesthetics thought to be membrane active are known to be inhibited by the simultaneous application of elevated pressure¹⁵, we suspected that thermal cell killing would also be inhibited by high pressure, as occurs in bacterial systems¹⁶. There are marked differences, however, between the response of mammalian cells and bacteria to heat: mammalian cells require only very small elevations in temperatures to effect large changes in survival, and can only grow within a comparatively narrow temperature range. We demonstrate here that pressure does, nevertheless, inhibit thermal killing of mammalian cells.

The survival of cells from a sub-line of Chinese hamster ovary (CHO) fibroblasts (HA-1) was measured by their ability to form clones after exposure to elevated temperatures for varying periods. During hyperthermic exposure, cells were

pressurized up to 3,000 p.s.i. (205 ATA) under a helium atmosphere. The survival of cells heated at 42.9 °C and 43.7 °C for up to 4 h is shown in Fig. 1. The application of pressure (2,500 and 3,000 p.s.i., respectively) increased survival by up to a factor of 10⁴. Control experiments showed no killing of cells incubated in identical conditions at 37 °C with or without pressure. The survival of cells heated at 44.0 °C for 90 min as a function of pressure is shown in Fig. 2. Their survival increases exponentially with increasing pressure over the range examined. Note that the degree of improved survival is actually underestimated by our technique because short periods of heating occurred before and after pressurization; also there was a small transient temperature elevation associated with the pressurization itself (see Fig. 1 legend).

We have demonstrated that the viability of heat-treated HA-1 cells, their ability to reproduce and form clones, is enhanced if pressure is applied simultaneously with the heat exposure. The most widely accepted theories of anaesthesia assert that general anaesthetics exert their effects by inducing a molecular volume expansion of cellular lipids or proteins^{15,19–22}. When the cell or organism is exposed to high pressure, molecular volume is reduced and anaesthesia, therefore, abolished. If this proposed mechanism is relevant, molecular volume expansion due to temperature elevation should also be pressure-reversible. As the volume expanding and fluidizing effects of both temperature elevation and anaesthetics on lipid bilayers and biomembranes are pressure-reversible^{23–25}, our observation of pressure reversibility of mammalian cell thermal killing suggests the cell membrane as a thermal target. However, pressure-reversibility does not in itself implicate solely the plasma membrane: the impairment of soluble enzyme activity by heat and anaesthetics has been shown to be pressure-reversible in the luciferin-luciferase enzyme system^{22,26}. An interesting question arising from this and the present findings is whether the phenotype of temperature-sensitive mutants at the restrictive temperature may be reverted to wild type by the application of high pressure.

The reversal of a temperature-induced increase in membrane fluidity by high pressure seems to have a biological analogue: when confronted by a change in environmental temperature,

cells induce compensatory alterations of membrane fluidity by changing their lipid composition²⁷⁻²⁹. We have recently found that CHO HA-1 cells increase their membrane cholesterol content and decrease membrane fluidity in response to elevated growth temperatures. Increased membrane cholesterol has also been associated with increased resistance to hyperthermic exposure (ref. 30 and R. Anderson *et al.*, in preparation). It has been proposed that cells may also adapt

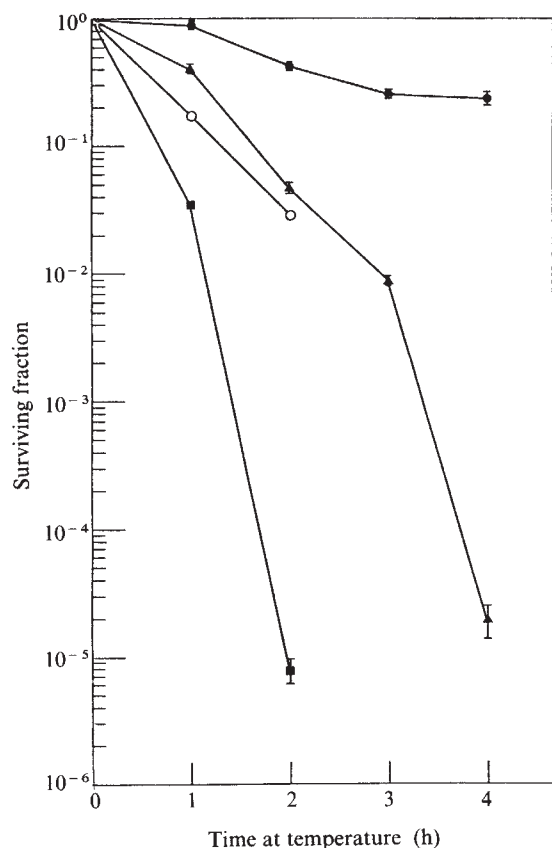


Fig. 1 The survival of HA-1 fibroblasts exposed to hyperthermia with and without pressure. HA-1 cells were grown for 3 days in 5 ml of minimal essential medium (MEM; Gibco) plus 15% fetal calf serum (FCS; Gibco) on the bottoms of gauze-plugged 50-ml volumetric flasks. Their growth was exponential with a final density of 80,000 cells cm^{-2} . Just before heating, fresh MEM plus 15% FCS was added to a total volume of 50 ml. The pressure bomb is as previously described¹⁷. It was filled with water and submerged in a temperature-controlled waterbath, and the system equilibrated to the desired temperature. The flask was submerged within the pressure bomb just to the end of the neck with the gauze plug above water level. Temperature equilibration of the media occurred within 5 min. The pressure bomb was equilibrated at 1 atm (14.7 p.s.i.) with 5% CO_2 and air. Helium was then added from a high pressure cylinder to achieve the desired pressure up to 3,000 p.s.i. Full pressure was achieved about 10 min after the flask was submerged. Depressurization and removal of the flask required 5 min. The pH of the medium was measured immediately before and after pressurization and remained constant at 7.6 ± 0.1 ; it was not affected by the degree or duration of pressurization. Temperature control was accurate to 0.1°C except for the period during and immediately after pressurization; pressurizing resulted in an elevation of temperature within the bomb and flask of 0.3 – 0.5°C which returned to baseline within 15 min. Survival was determined according to the cloning assay of Puck and Marcus¹⁸. ▲, 42.9°C , unpressurized; ●, 42.9°C , 2,500 p.s.i.; ■, 43.7°C , unpressurized; ○, 43.7°C , 3,000 p.s.i. Bars indicate standard error.

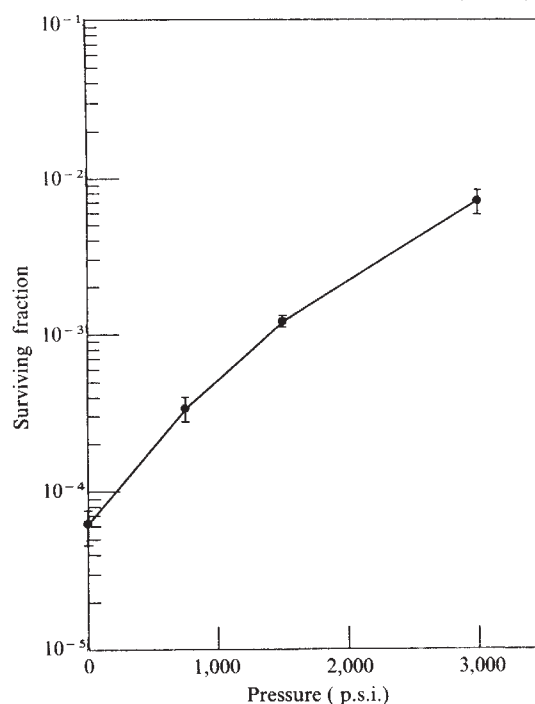


Fig. 2 The survival of HA-1 fibroblasts exposed to 44.0°C for 90 min at pressures ranging up to 3,000 p.s.i. See Fig. 1 legend for procedure. Bars indicate standard error.

to anaesthetics by altering membrane composition³¹. This has been confirmed recently in the case of ethanol where it has been demonstrated that membranes from ethanol-tolerant mice show increased cholesterol and increased resistance to the fluidizing effect of ethanol^{32,33}.

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Transformation of rat fibroblasts and formation of virus-induced syncytia

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Human KC cells¹ and rat XC cells² transformed by Rous sarcoma virus (RSV) fuse to form multinucleate syncytia in the presence of several retroviruses³⁻¹¹, although neither cell line itself produces virus. XC cells form syncytia on co-cultivation with cells producing murine leukaemia virus (MuLV)³ and with purified MuLV⁴. This altered cytopathic property is readily quantified and is a useful assay both for titrating MuLV and for detecting cells producing these viruses^{3,5,6}. Fusion occurs from without and does not require viral replication⁷. We recently described the fu-1 cell line of developmentally defective rat myoblasts, which can also undergo syncytia formation either by co-cultivation with cells infected with Moloney MuLV (MoMuLV) or on infection with intact or UV-inactivated MoMuLV¹². On chronic infection with MoMuLV, fu-1 cells become refractive to syncytia formation¹²; this is also true of XC and KC cells infected with their appropriate respective viruses^{9,11,13}. However, in contrast with XC and KC cells, fu-1 cells do release an endogenous retrovirus^{14,15}. Whereas XC, KC and fu-1 cells are transformed and can undergo virus-induced fusion, neither 118 MG cells¹⁰ nor L₈ myoblasts¹² (the non-transformed parents of KC and fu-1 cells, respectively), form these virus-induced syncytia. This suggests that the capacity of cells to undergo retrovirus-induced fusion rests with their transformed status. We report here that Rat-1 cells, transformed with the temperature-sensitive transformation mutant RSV-tsLA29 (ref. 16), only form syncytia in the presence of MoMuLV when these cells are preincubated at 35°C, the temperature permissive for transformation, and not at 40°C, the non-permissive temperature. Rat-1 cells transformed with wild-type avian sarcoma virus form MoMuLV-induced syncytia at both temperatures. Thus, virus-induced transformation promotes changes in these cells that render them competent to undergo subsequent virus-induced fusion.

Rat-1-tsLA29 and fu-1 cells were grown for 18 h at either 35°C or 40°C and were then infected with MoMuLV and incubated for an additional 5 h at either temperature. MoMuLV was effective at inducing syncytia in fu-1 cells at either temperature, although the number of syncytia formed at 40°C was slightly less than that formed at 35°C (Table 1, expt 1). In contrast, only those Rat-1-tsLA29 cells preincubated at 35°C fused in the presence of MoMuLV (Table 1 and Fig. 1). Although shifting the temperature from 35 to 40°C, either at the time of infection or during the 5-h post-infection period, reduced the number of syncytia that developed, a shift from 40 to 35°C at either of these times did not promote cell fusion. Thus, the capacity of these cells to undergo virus-induced fusion clearly depends on the expression of the temperature-sensitive transforming properties of the virus and this competence must be present during the initial interaction of the cells and virus.

Rat-1 cells not transformed by avian sarcoma virus and Rat-1 cells transformed with wild-type B77 avian sarcoma virus (Rat-1-B77 cells) were also tested for their capacity to undergo virus-induced fusion (Table 1, expt 2). MoMuLV did not induce the fusion of normal Rat-1 cells to an appreciable extent at either temperature. Rat-1-B77 cells, like fu-1 cells, fused at both 35 and 39.5°C. Thus, fusion induced by MoMuLV is not inherent in Rat-1 cells but is dependent on the virus-induced transformation of these cells.

We have previously shown that several developmentally defective clones isolated from the L₈ myogenic line can undergo MoMuLV-induced fusion and that this capacity to undergo virus-induced fusion is significantly associated with several phenotypic traits characteristic of transformed cells *in vitro*^{14,15}. These changes include elevated hexose transport, decreased cell-surface fibronectin, reduced serum requirements for growth and decreased adhesiveness. These are also characteristics previously reported to be associated with expression of the *src* gene of RSV. Whether any of these changes in the cell surface are causally related to the capacity of these cells to undergo retrovirus-induced fusion is of interest. Both normal and transformed cells can support productive infections by MoMuLV and this suggests that both cell types can interact with the virus. In contrast, expression of the RSV transformation gene in Rat-1, KC and XC cells, and by analogy, expression of endogenous transformation sequences in fu-1 cells, is required and mediates the competence of these cells to undergo virus-induced fusion. Whereas leukaemia virus-induced fusion has proved to be a

Table 1 MoMuLV-induced fusion of fu-1 myoblasts and avian sarcoma virus transformed Rat-1 fibroblasts

Cells	Temperature (°C)			Syncytia per cm ²	
	Pre-infection (18 h)	Infection (1 h)	Post-infection (5 h)	+ MoMuLV	- MoMuLV
Expt 1 fu-1	35	35	35	6,376 ± 1,185	58 ± 115
	40	40	40	4,956 ± 973	28 ± 67
	35	35	35	3,009 ± 814	29 ± 68
	40	40	40	14 ± 49	0 ± 0
	35	35	40	1,734 ± 566	103 ± 139
	35	40	40	1,044 ± 283	14 ± 49
	35	40	35	1,274 ± 814	0 ± 0
	40	40	35	58 ± 60	14 ± 49
	40	35	40	28 ± 67	14 ± 49
	40	35	35	14 ± 49	44 ± 79
Expt 2 fu-1	35	35	35	1,120 ± 275	0 ± 0
	39.5	39.5	39.5	1,075 ± 183	0 ± 0
	35	35	35	51 ± 53	4 ± 16
	39.5	39.5	39.5	43 ± 38	0 ± 0
	35	35	35	595 ± 147	51 ± 70
	39.5	39.5	39.5	564 ± 126	16 ± 28
	35	35	35	595 ± 147	51 ± 70
	39.5	39.5	39.5	564 ± 126	16 ± 28

Rat-1-tsLA29 cells were derived as follows: viral pseudotypes were produced by infection of chick embryo cells with subgroup D chicken leukaemia virus (Carl Zilber Associated Virus) and RSV-tsLA29 (subgroup A). Rat-1 cells, a line of Fischer rat embryo cells (referred to as F2408 by Mishra and Ryan¹⁷ and Prasad, Zouzas and Basilico¹⁸) were infected with the pseudotype virus; 2 weeks later foci were isolated and grown up, and the Rat-1-ts LA29 cells were cloned. At 35°C, but not at 40°C, these cells exhibit morphological and growth control properties of transformed cells *in vitro*, including elevated hexose transport, enhanced colony formation in soft agar, increased proteolysis and decreased adhesiveness (M. Weber, personal communication). Rat-1-B77 cells (clone B1) were derived by an analogous procedure from the 208F subclone of Rat-1 cells¹⁹, using B77 avian sarcoma virus. Rat-1, Rat-1-tsLA29, Rat-1-B77 and fu-1 cells were grown and passaged as previously described^{14,15}, in Dulbecco's medium supplemented with 10% horse serum, 100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin and 10 µg ml⁻¹ kanamycin. 4 × 10⁵ fu-1, 4 × 10⁵ Rat-1, 8 × 10⁵ Rat-1-B77, or 1 × 10⁶ Rat-1-tsLA29 cells were plated on 35-mm Falcon tissue culture dishes. These different numbers of cells were plated to obtain subconfluent monolayers needed for optimal fusion¹². The cultures were incubated for 1 h at 37°C, then for 18 h at either 35°C or 40°C (39.5°C, expt 2). The medium was removed and the cells were infected with MoMuLV in 0.6 ml serum-free medium, containing 12 µg ml⁻¹ DEAE-dextran, at a multiplicity of infection of approximately 25. Control cultures were treated with the same medium, but without virus. After 1 h at 35°C or 40°C (39.5°C, expt 2), the virus was removed and the cells were washed with complete medium and incubated for 5 h at the temperatures indicated. The cells were then fixed in 70% methanol for 20 min and stained with 5% Giemsa. Cells containing three or more nuclei were scored as syncytia in at least six fields on each plate, at a magnification of ×160. The mean numbers of syncytia per cm² ± s.d. in duplicate plates are given. Expts 1 and 2 were done with different lots of MoMuLV.

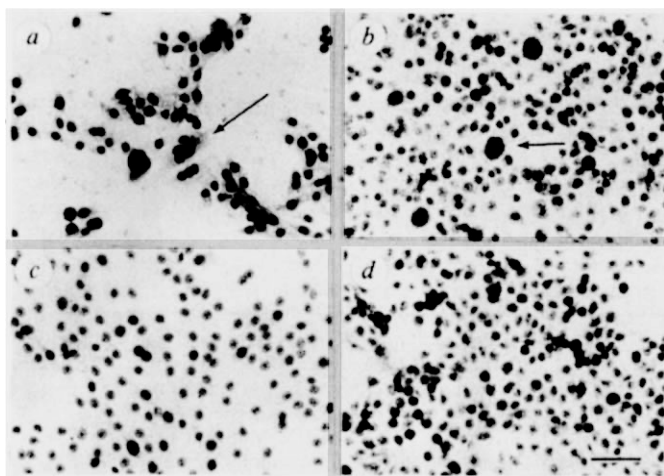


Fig. 1 Virus-induced syncytia in transformed Rat-1 cells. Syncytia were formed by infection of Rat-1-tsLA29 cells with MoMuLV as indicated in Table 1. Cells preincubated at 35°C and maintained at 35°C throughout the experiment (a), or transferred to 40°C on infection and post-incubation (b), formed MoMuLV-induced syncytia. Cells preincubated at 40°C and maintained at 40°C during and after infection (c) did not form syncytia; similarly, cells preincubated and maintained at 35°C did not form syncytia in the absence of MoMuLV (d). Scale bar, 50 µm.

useful assay for quantifying these viruses and for detecting cells producing these viruses, the results presented here suggest that MoMuLV-induced fusion may also be useful as a rapid assay for cells that have undergone virus-induced or 'spontaneous' transformation.

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2'5'-oligo(A) polymerase activity in serum of mice infected with EMC virus or treated with interferon

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Interferon-treated cells show an increase in two double-stranded RNA (dsRNA)-dependent enzymatic activities involving an oligoadenylate polymerase and a protein kinase (ref. 1 and refs therein). The polymerase converts ATP into a series of oligonucleotides characterized by 2'5'-phosphodiester bonds, designated 2'5'-oligo(A) or 2-5A (ref. 1). These oligonucleotides activate an endoribonuclease that degrades RNA¹ in extracts of control and interferon-treated cells. These observations have been made in tissue culture cells and no information is yet available on these enzymatic activities in animals with elevated interferon levels. We report here on 2-5A synthesis in tissue homogenates and serum of mice infected with encephalomyocarditis virus (EMCV); this virus induces interferon synthesis when injected intraperitoneally into mice². Significant synthesis of 2-5A was detected in extracts of spleen and lungs, but also, surprisingly, in the serum of these mice. Subsequent experiments showed synthesis of 2-5A in serum of mice treated with the interferon inducer poly(I)·poly(C) (ref. 3) or with mouse fibroblast interferon.

Male CD mice were injected intraperitoneally (i.p.) with different doses of EMCV and killed 3 days after infection. The mice were bled to obtain serum, and tissue extracts were prepared from the spleen, lungs, liver and kidney as described in Fig. 1 legend. Synthesis of 2-5A was assayed in incubations containing poly(I)·poly(C) and ³H-ATP; this assay and the separation of 2-5A from ATP by DEAE-cellulose chromatography have been previously described⁴. The assay requires at least 1% conversion of ATP into 2-5A for polymerase to be unambiguously detected. Significant synthesis of 2-5A was observed with serum, whereas lesser amounts of 2-5A were synthesized with spleen and lung extracts; no significant synthesis of 2-5A was obtained with

other tissue extracts (data not shown). The synthesis of 2-5A with serum and spleen or lung extracts was related to the dose of infecting virus, suggesting a relationship between the severity of infection and the 2-5A synthetic activity (Fig. 1a).

The failure to observe synthesis of 2-5A with liver and kidney extracts could be explained either by the absence of 2-5A synthetic activity or by the presence in these tissues of other enzymatic activities which may interfere with the activation of 2-5A polymerase or with the accumulation of 2-5A. Therefore, we assayed the extracts for degradation of poly(I)·poly(C), the activator of 2-5A polymerase, and for degradation of A2'p5'A (Table 1). An RNase which degrades dsRNA has been found in mammalian cells⁵⁻⁷ and a phosphodiesterase activity that cleaves 2'-5' bonds has also been reported in extracts of tissue culture cells⁸⁻¹⁰. Degradation of poly(I)·poly(C) was observed with all the tissue extracts tested, but not with mouse serum.

The activation of 2-5A polymerase requires perfectly matched dsRNA more than 30 base pairs long and degradation of dsRNA by RNase may prevent synthesis of 2-5A (ref. 11). Because poly(I)·poly(C) was not degraded by mouse serum, in the following experiments we assayed synthesis of 2-5A with serum samples; we also assayed spleen extracts because of the significant accumulation of 2-5A, shown in Fig. 1. Accurate measurement of 2-5A synthesis in tissue extracts, however, requires at least partial purification of 2-5A polymerase activity, for example by adsorption to poly(I)·poly(C) bound to agarose¹² or to paper¹³. Using the paper assay Stark *et al.*¹³ established the wide distribution of 2-5A polymerase in a variety of cells and tissues and demonstrated its variation with growth and changes in hormonal status. They used the much more sensitive biological assay for 2-5A to detect the polymerase¹³. We assayed liver and kidney extracts from control and infected mice after adsorption to poly(I)·poly(C)-agarose¹⁴, but could not detect significant synthesis of 2-5A with extracts from control mice or liver of infected mice, but synthesis of 2-5A above background level was observed with extract of kidney from infected mice. It should be pointed out, however, that this assay is relatively insensitive when compared with the biological assay and that synthesis of 2-5A could possibly be detected by the paper assay in all tissues.

Phosphodiesterase activity that cleaves A2'p5'A was detected in all samples tested (Table 1). This activity, however, is inhibited by ATP present in the assay for 2-5A synthesis¹⁰. This was established by adding ³H-2-5A synthesized as described¹⁰ to an incubation containing 5mM ATP and mouse serum but not poly(I)·poly(C). Less than 5% of the 2-5A was degraded after 90 min, as determined by

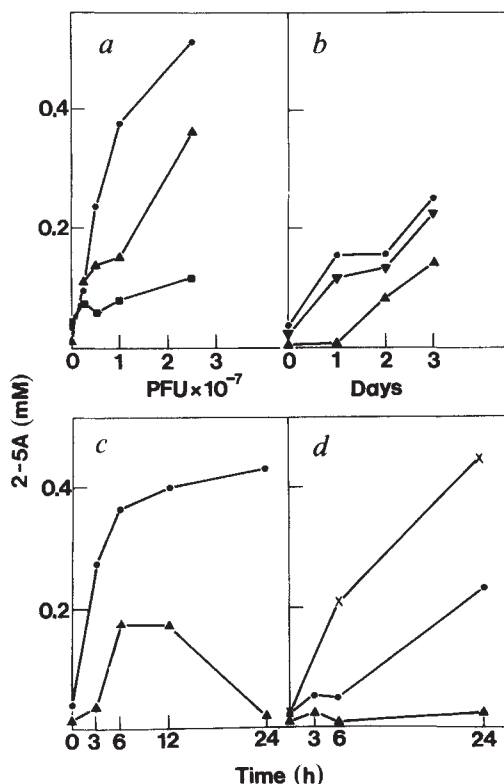


Fig. 1 Synthesis of 2-5 oligo(A) with serum (●, ×) or plasma (▼), and extracts of spleen (▲) or lungs (■) of mice infected with different doses of EMC virus for 3 days (a), infected with 5×10^6 PFU of EMCV for different times (b), injected with 0.1 mg of poly(I)·poly(C) complexed with 0.1 mg of DEAE-dextran (c) and injected with 10^5 U (×) or 10^4 U (●, ▲) of mouse fibroblast interferon (d). EMCV was grown in Ehrlich ascites tumour cells and plaque-assayed on L-cell monolayers. Virus stocks were diluted to 0.5 ml with phosphate-buffered saline, pH 7.2, and injected i.p. in CD mice. Poly(I)·poly(C) and interferon were also diluted to 0.5 ml with saline and injected in the same way. At the indicated times the mice were anaesthetized and bled from the brachial artery to obtain serum or plasma on addition of 3 mg ml^{-1} of sodium citrate. The spleen and lungs were then excised and washed with 10 mM KCl, 1.5 mM MgCl_2 , 0.5 mM dithiothreitol and 20 mM HEPES-KOH buffer, pH 7.4. Extracts were prepared by homogenization of 1 g of tissue with 2 ml of this buffer in a motor-driven Potter homogenizer and centrifugation for 5 min at 30,000g. The supernatant, serum and plasma were stored at -70°C . Synthesis of 2-5A was assayed in 25- μl reactions incubated for 90 min at 30°C and containing: 5 μl of extract or serum, 0.25 μg of poly(I)·poly(C), 125 nmol of ³H-ATP (about 0.2 μCi), and the other components previously described⁴. The reactions were stopped by heating to 90°C for 3 min and applied to $0.5 \times 0.5 \text{ cm}$ DEAE-cellulose columns; ATP was eluted with 90 mM KCl and 2-5A with 0.35 M KCl, as described⁴. As a control, the nucleotides eluted with 0.35 M KCl were diluted to 90 mM KCl and reappplied to DEAE-cellulose columns; the recovery after washing and elution with 0.35 M KCl was better than 80%. The ordinate shows the concentration of ATP converted into 2-5A. Analysis of reactions without poly(I)·poly(C) or kept at 0°C gave $<700 \text{ c.p.m.}$ eluted with 0.35 M KCl out of an input of $\sim 125,000 \text{ c.p.m.}$ This background was subtracted from the data shown; it corresponds to $5.6 \mu\text{M}$ ATP.

Table 1 Degradation of poly(I)·poly(C) and A2'p5'A by tissue extracts and serum

Tissue	Poly(I)· ³ H-poly(C) (% soluble)	A2'p5'A (nmol degraded)
Spleen	92	20.4
Lungs	35	4.5
Liver	59	11.2
Kidney	43	29.2
Serum (mouse)	1	17.7
Serum (human)	86	34.5

Tissue extracts and serum were obtained from mice infected for 3 days with 1×10^7 plaque-forming-units (PFU) of EMCV as described in Fig. 1 legend; the human serum was from a healthy donor. Assays for dsRNA degradation contained: 25 μl of tissue extract or serum and 5 μg of poly(I)·³H-poly(C) (43 nCi μg^{-1} ; Miles) in a final volume of 75 μl . After 60 min at 30°C , the samples were made 5% in trichloroacetic acid and the undigested dsRNA collected on filters for counting. Per cent soluble is expressed relative to a sample not incubated. Assays for A2'p5'A degradation contained: 80 nmol of dinucleotide, 10 μl of tissue extract or serum, 0.12 M K(OAc), 20 mM $\text{Mg}(\text{OAc})_2$, 1 mM dithiothreitol, 20 mM HEPES-KOH, pH 7.4, in a final volume of 50 μl . After 90 min at 30°C the reactions were diluted to 0.5 ml with 2.5 mM Tris-HCl, pH 7.4, and passed through $0.5 \times 0.5 \text{ cm}$ columns of DEAE-cellulose equilibrated with the same buffer. Adenosine was washed from the column with another 0.5 ml of buffer. The breakthrough and wash fractions were combined and absorbance at 260 nm measured. Reactions with no added A2'p5'A were run at the same time to obtain blank values, which were subtracted to calculate nmol of adenosine formed.

chromatographic analysis on DEAE-cellulose columns in the presence of 7 M urea (see below). Therefore, synthesis of 2-5A could be measured with mouse serum samples, since neither the activator nor the reaction product was appreciably degraded during incubations.

The synthesis of 2-5A with serum was unexpected, as 2-5A polymerase activity had previously been shown only in cell extracts¹. Therefore, several experiments were carried out to characterize the presumptive 2-5A synthesizing activity in serum and the products of the reaction. The oligonucleotides synthesized with serum were identified as 2-5A by TLC (not shown) and DEAE-cellulose columns (Fig. 2) before and after digestion with nuclease and alkali, following previously described procedures^{4,15}. The major reaction product had a net charge of about -4.6 and was eluted before a minor peak of charge about -5.6 . These oligonucleotides were not digested by T2 RNase, which cleaves 3'-5' phosphodiester bonds, but were digested by snake venom phosphodiesterase to AMP, by alkali to adenosine and a nucleotide which eluted with marker p5'A2'p (Fig. 2b), and by alkaline phosphatase to products which eluted with the same charge as A2'p5'A and A2'pA2'p5'A, respectively (Fig. 2c). In this last experiment we analysed the products of a 17-h incubation with serum; a large amount of the minor component was present in this sample.

These data and TLC results indicate that the nucleotides synthesized with serum are a di- and trinucleotide containing adenosines linked by 2'-5' phosphodiester bonds and a terminal triphosphate, as shown by the -4 charge loss on digestion with phosphatase (Fig. 2c). Furthermore, when tested for biological activity, the oligonucleotides synthesized in a 17-h incubation with serum activated an endonuclease similarly to previously characterized 2-5A (ref. 16). This was established by adding 0.2 μM presumptive 2-5A to a HeLa cell extract containing labelled viral mRNA, and measuring mRNA cleavage¹⁶. About 80% of the viral mRNA was cleaved in a 2-h incubation, whereas insignificant cleavage was observed with the products of a reaction containing serum of control mice (data not shown). The synthesis of these oligonucleotides was: (1) dependent on the addition of dsRNA (see Fig. 1 legend); (2) optimal with Mg^{2+} concentrations higher than 10 mM;

and (3) proportional to ATP concentration up to 7 mM. The 2-5A polymerase activity present in extracts of interferon-treated cells shows similar characteristics⁴.

In subsequent experiments, synthesis of 2-5A was assayed at different times after infection with a standard dose of EMCV, and after injection of poly(I)·poly(C) or mouse fibroblast interferon (Fig. 1). Serum obtained 1 day after injection with EMCV showed significant synthesis of 2-5A; serum samples obtained later in the infection synthesized greater amounts of 2-5A (Fig. 1b). Plasma prepared with sodium citrate as anticoagulant and extracts of spleen obtained from mice infected for 2 or 3 days also synthesized 2-5A. These results indicate that during EMCV infection progressively higher levels of 2-5A polymerase activity may be present in the serum and spleen. Further experiments established that the presence of this enzymatic activity could be explained by the induction of interferon synthesis.

Mice were injected with poly(I)·poly(C) complexed with DEAE-dextran, as the latter strongly enhances the interferon¹⁷ synthesis. Serum obtained 3–24 h after injection of the complex actively synthesized 2-5A (Fig. 1c), but serum obtained from mice 30 min, 1 h and 2 h after injection of the complex did not synthesize significant amounts of 2-5A (data not shown). Spleen extracts were most active 6 h after injection. The synthesis of 2-5A with serum obtained 3 h after poly(I)·poly(C) injection was confirmed in subsequent repeated experiments. This may be explained by the increase in circulating interferon in mice 1.5 h after injection of poly(I)·poly(C)/DEAE-dextran; the interferon titre reaches a maximum at about 3 h (ref. 17).

The serum of mice injected with mouse interferon also synthesized 2-5A (Fig. 1d). The serum of mice injected with 10^5 U interferon showed greater activity than that of mice injected with 10^4 U, but the increase in activity with time was slower than that observed in mice injected with poly(I)·poly(C) and spleen extracts were not active. It seems possible that greater levels of circulating interferon were present in mice injected with poly(I)·poly(C) than in mice receiving a single i.p. injection of fibroblast interferon; this remains to be shown, however.

In conclusion, the serum from EMCV-infected mice and from animals injected with poly(I)·poly(C) or interferon contains an enzymatic activity similar to that characterized in interferon-treated tissue culture cells. The products of incubations with serum are predominantly dimer and trimer, whereas extracts of tissue culture cells synthesize higher oligomers in optimal conditions⁴. This pattern of 2-5A synthesis may be characteristic of serum, although an overnight incubation was also necessary to obtain significant synthesis of trimer with L-cell extracts in some assay conditions¹⁸. Synthesis of 2-5A can also be shown in some tissues, although degradation of the activator dsRNA by tissue extracts interferes with the assay.

The presence of 2-5A polymerase activity in serum is puzzling. It cannot be due to the release of cytoplasmic enzymes from cells lysed by infecting EMCV, as a similar activity is found after interferon injection. It seems possible, however, that 2-5A polymerase activity may be released from interferon-treated cells and from cells treated with poly(I)·poly(C). The biological role of the 2-5A polymerase activity in serum, if any, is unknown. Synthesis of 2-5A with serum, however, may be indicative of a viral infection. Attempts to measure 2-5A synthesis in serum of individuals affected by viral diseases have so far failed (unpublished observations) because of the very high levels of nucleases that degrade dsRNA in human serum (Table 1 and refs 19–21). Further experiments are in progress to investigate the presence of 2-5A polymerase in human serum and the release of this enzymatic activity from cells. As Williams *et al.*²² have reported the natural occurrence of 2-5A in interferon-treated mouse L cells infected with EMCV, it was of considerable

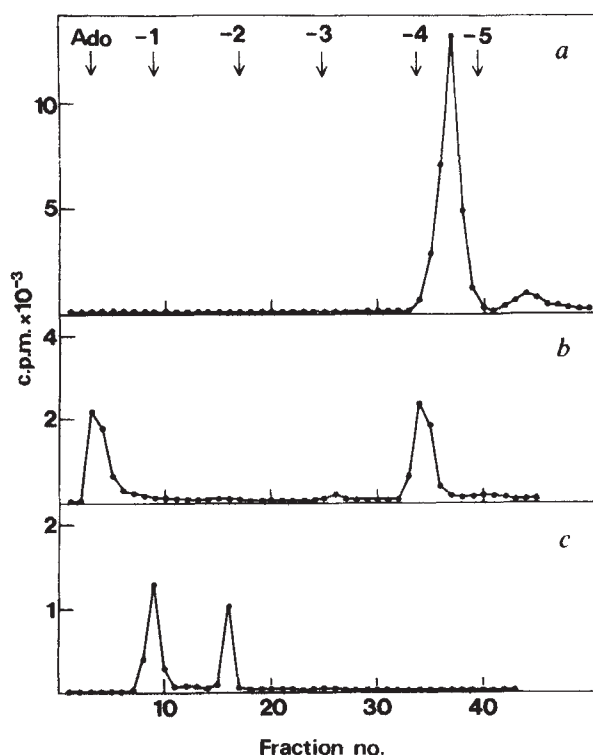


Fig. 2 Chromatographic analysis on DEAE-cellulose columns of the reaction products of serum obtained from mice either 3 days after injection with 2.5×10^7 PFU of EMCV or after injection with poly(I)·poly(C) complexed with DEAE-dextran, and incubated with poly(I)·poly(C) and 25 μ Ci of 3 H-ATP as described in Fig. 1 for 2 h (a and b) or for 17 h (c). Essentially identical results were obtained with both samples. The oligonucleotides synthesized were first adsorbed to small DEAE-cellulose columns and eluted with 0.35 M KCl⁴. a, A 10- μ l sample was diluted to 1 ml with 50 mM NaCl, 7 M urea, 20 mM Tris-HCl, pH 7.6 and applied to a 0.5×25 cm column of DEAE-cellulose equilibrated with the same buffer. Unlabelled markers (Sigma) were added with the sample: adenosine (Ado), A2'p5'A (charge -1), AMP (-2), ADP (-3), p5'A2'p (-4) and p4A (-5). The nucleotides were eluted through a recording spectrophotometer with a linear 300-ml gradient from 50 to 300 mM NaCl in the buffer described above; 3-ml fractions were collected and 1 ml counted. The position of markers is indicated by their charge, except for Ado; in separate runs ATP was eluted a few fractions before p5'A2'p. b, The sample shown in a was incubated for 17 h in 0.3 M KOH before chromatography. c, The oligonucleotides were digested with alkaline phosphatase as described⁴ before chromatography. In control chromatographic analysis ATP incubated with KOH or phosphatase yielded AMP and adenosine respectively. Treatment with KOH, therefore, hydrolyses the β and γ phosphates.

interest to establish whether 2-5A itself is present in serum. Preliminary experiments were carried out with sera from control mice and from mice treated as indicated in Fig. 1. Aliquots of serum (25 μ l) were heat-treated and the supernatant obtained from centrifugation was chromatographed on DEAE-cellulose to obtain a fraction eluting with 1 M NH_4HCO_3 (ref. 22); after extensive lyophilization this fraction was assayed for nuclease activation in HeLa cell extracts¹⁶. Nuclease activity was present in all the samples prepared in this way; therefore, these samples could not be tested in the assay for 2-5A-dependent endonuclease¹⁶. The presence of 2-5A in serum thus remains to be established, possibly by a more extensive purification of serum oligonucleotides²².

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Antibodies to a neural cell adhesion molecule disrupt histogenesis in cultured chick retinae

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Cell-surface proteins are believed to have important roles in cell-cell interactions during brain development, particularly in such processes as cellular adhesion, neurite outgrowth and synapse formation¹⁻⁴. The chick neural cell adhesion molecule, CAM, is a cell-surface protein specific to the nervous system and has been implicated in cell adhesion among cells and neurites of the developing retina and brain⁵⁻⁸. Previous studies have shown that F(ab') fragments of antibodies directed against CAM inhibit the *in vitro* aggregation of cells obtained from 9-day embryonic chick retina. The specific antibody fragments also reduce the diameter of neurite fascicles that grow out from cultured dorsal root ganglia, apparently by blocking side-to-side adhesion between the neurites. In addition, anti-CAM antibodies alter the appearance of histotypic patterns in retinal cell aggregates maintained in culture for several days⁷. We now demonstrate that the antibodies can disrupt histogenesis of the developing retina in organ culture, strengthening the notion that the cell-cell adhesion properties mediated by CAM are involved in the normal development of histological layers in the chick retina.

In the chick, cell and plexiform layers of the central portion of the retina are formed between the sixth and ninth days of embryonic life^{9,10}. Although many cells have left the mitotic cycle by day 6 (ref. 9), all remain in a single nuclear or 'matrix'

layer. Retinae dissected from White Leghorn embryos on day 6 and placed in organ culture for 3 days undergo histogenesis similar to that which occurs *in vivo*, as can be seen in Fig. 1. To achieve uniformity of developmental stage, all retinal fragments were dissected from the region surrounding the choroid fissure. Pigment epithelium clinging to the neural retina was largely removed; any remaining pieces of epithelium seem not to have altered the progress of development. Retinal fragments were placed with their vitreous side down on 13-mm Millipore filters, 1.2 µm pore size, that had been wetted with medium, and were cultured on the stainless steel grid of a Falcon organ culture dish. In all experiments the medium was Dulbecco's modified Eagle's medium, supplemented with 1/10 volume of heat-inactivated fetal calf serum (Microbiological Associates). The anti-CAM sera were obtained from rabbits injected with purified CAM as described earlier⁵, and control antisera were obtained from unimmunized rabbits. F(ab') fragments prepared from the antibodies were dissolved in the tissue culture medium to a concentration of 1 mg ml⁻¹. All cultures were carried out for 3 days at 37°C in an atmosphere of 5% CO₂, and the retinae were then fixed either in Bouin's fixative for paraffin embedding or for 1 h in 2% glutaraldehyde in phosphate-buffered saline in preparation for embedding in Epon 812.

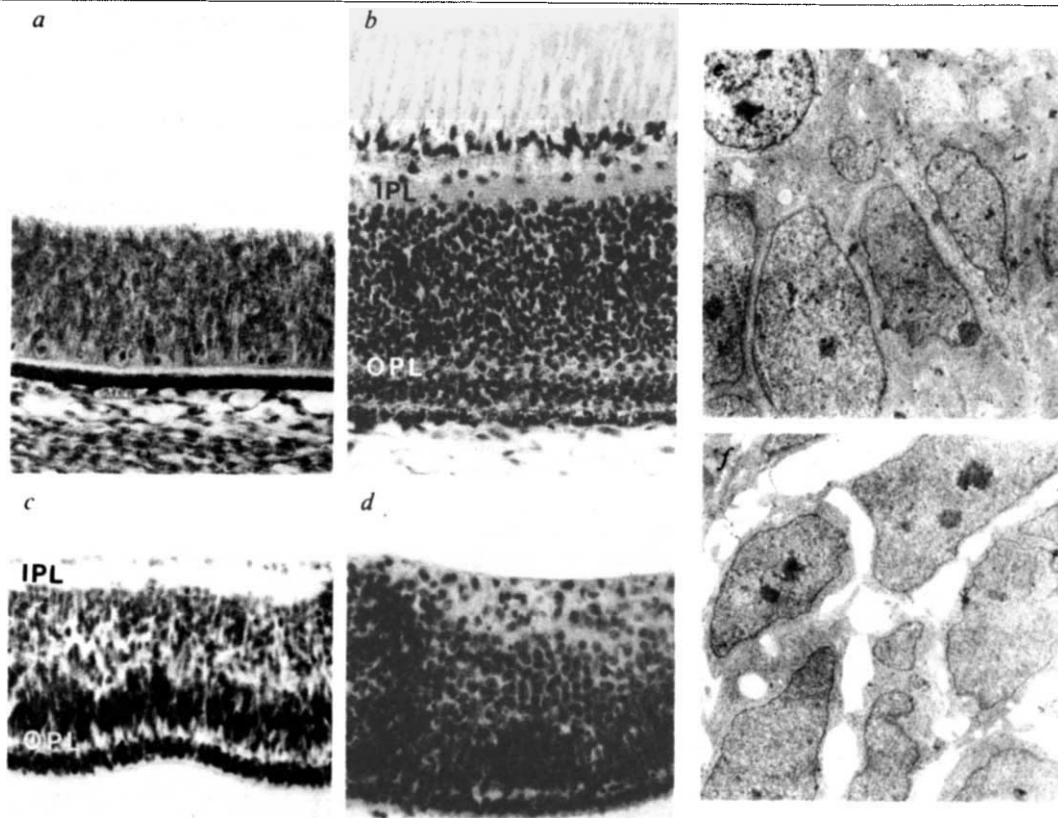
Tissues cultured in the presence of anti-CAM showed both disorder in the pattern of histological layers and an increase in the extracellular space surrounding the cell perikarya. In contrast, retinae cultured in the presence of antibodies from unimmunized rabbits developed in a manner analogous to retinae *in vivo*.

Paraffin sections of retinae cultured with non-immune serum (Fig. 1) revealed that, during the 3 days of culture, the initially rather uniform nuclear layer had developed distinct histological layers resembling the retina of a 9-day-old embryo. Inner and outer plexiform layers were clearly visible, as were the layers of photoreceptor cell bodies and cells believed to be ganglion cells. The photoreceptor cells had buds which mark the first stage in the development of the inner segments. The substantial layer of ganglion cells found in the *in vivo* 9-day retina was not observed, however, in the cultured retinae. Other workers studying the differentiation of chick retinae transplanted to the chorioallantoic membrane also observed a deficiency of ganglion cells, and attributed this to a failure to make tectal connections¹¹. It is also possible that the cells observed along the vitreous edge of cultured retinae represent displaced ganglion cells which during the normal course of development are migrating back to the nuclear layer^{12,13}.

Retinal tissues cultured in the presence of anti-CAM displayed several additional alterations which sharply reduced the resemblance to normal retina. The ganglion cells, normally arranged in an even layer along the vitreous edge of the retina, were less organized and were found scattered throughout the inner plexiform layer. In control retinae, cells within the nuclear layer could be divided into two layers based on cell shape, the outermost consisting of bipolar and horizontal cells and the layer towards the vitreous side of the nuclear layer corresponding to the amacrine cells. In the retinae exposed to anti-CAM (Fig. 1e), these layers were not clearly distinguishable, suggesting that the distribution of specific cell types had been altered. More definitive evidence on the exact arrangement and morphological differentiation of specific cell types will require additional studies using Golgi staining techniques.

Electron microscopy of the anti-CAM-treated tissues revealed differences in the packing of cells in the nuclear layer. In the tissue cultured with anti-CAM F(ab') fragments, large extracellular spaces surrounded the cell bodies of the nuclear layer. The extracellular spaces in the retinae cultured without anti-CAM F(ab') were similar to those observed in sections of *in vivo* 9-day retinae. This difference was observed in all tissues

Fig. 1 *a, b* Represent *in vivo* chick retinae at 6 and 9 days of embryonic age, respectively. Retinae are from the central, most differentiated area of the retinae. The scleral side of the retinae, containing the photoreceptor cells, is shown towards the bottom in these photographs; in both photographs the pigment epithelia and scleral tissue can be seen lying below the neuroretina. In *b* the outer and inner plexiform layers are clearly visible. *c, d* Are chick retinae that have been in organ culture for 3 days following their dissection from the embryo on day 6. The retina shown in *c* was cultured in the presence of F(ab') antibody fragments from unimmunized rabbits, and that in *d* in the presence of antibodies to the cell adhesion molecule CAM. Although the cultured retinae are thinner and not as fully developed as *in vivo* 9-day retinae, the histological layers can be clearly identified and mark a significant differentiation from the 6-day stage. The retinae cultured in the presence of anti-CAM showed identifiable but disrupted histological layers which do not have the sharp boundaries between adjacent layers characteristic of both the *in vivo* and the cultured control retinae. Ganglion cells, at the top of the photographs, form neat rows in the *in vivo* and control retinae, but are scattered in the retinae treated with anti-CAM. The light micrographs are $\times 650$. *e, f* Are electron micrographs of cell bodies on the vitreous side of the nuclear layer of retinae cultured in the presence of non-immune and anti-CAM F(ab'), respectively. Note that the cells in the control retina show large areas of membrane apposition and small areas of extracellular space, whereas the retina cultured with antibodies to CAM shows large areas of extracellular space and few areas of cell-cell contact. Electron micrographs are $\times 5,800$.



sectioned, including those which had differentiated poorly during the culture period. It resembles changes previously seen in histotypic aggregates that were similarly treated⁷. The possibility that this difference is a consequence of increased susceptibility of the anti-CAM-treated retinae to shrinkage during processing has not been ruled out completely, but seems unlikely in view of the normal appearance of cytoplasmic ultrastructure in the treated retinae. This question is being investigated directly through the measurement of extracellular space by horseradish peroxidase diffusion and staining techniques.

As judged by dense haematoxylin staining, necrotic cells were present in both untreated cultured retinae and those treated with anti-CAM. Approximately 1% of the cells in the treated retinae were pycnotic at day 9, and about 0.1% of the untreated retinal cells also seemed to be pycnotic. The amount of total cell loss during the 3-day period is not known, but both control and anti-CAM retinae were thinner than in *in vivo* 9-day retinae.

The present results extend the previous studies on CAM by demonstrating that antibodies against CAM disrupt the internal development of a tissue never subjected to mechanical dissociation. The observed increase in the extracellular space and the concomitant decrease in regions of cell-cell contact are consistent with the interpretation that CAM is a cell adhesion molecule. Because the individual cells in the anti-CAM-treated retinae were morphologically similar to those in the control tissues, it seems that the effects are not simply a consequence of changes in the developmental pathway of

individual cells. This conclusion is supported by the ability of the anti-CAM-treated retinae to form some histological layers which, although altered, could be directly identified with layers in the normal retina. It seems, then, that the processes necessary for the differentiation of cell types and the formation of plexiform layers may occur unimpeded by the presence of anti-CAM. Nonetheless, the observation of misplaced ganglion cells and the apparent mixing of cell types in the nuclear layer suggests that, during the 6–9-day period of embryonic retinal development, cell-cell interactions mediated by CAM are important in achieving the appropriate arrangement of cells in nervous tissue.

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Absence of M protein in a cell-associated subacute sclerosing panencephalitis virus

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Measles virus has been suggested to cause subacute sclerosing panencephalitis (SSPE), a slow central nervous system disease of children. However, several questions remain about the pathogenesis of SSPE. For example, it is not known whether alteration of the measles virus genome has a role in the initiation and persistence of the disease. Several studies have compared the RNA and protein composition of wild-type (wt) and SSPE strains of measles virus in a search for markers characteristic of the latter¹⁻⁴. All the studies used SSPE strains that had reverted to the budding, virion-producing form, similar to wt. We have shown, however, that only cell-associated non-budding strains of SSPE virus cause an SSPE-like persistent infection in young ferrets⁵. Strong cell association and cell-fusing activity were essential for the virulence of measles virus in the brains of experimental animals and possibly humans. We have, therefore, compared the protein composition of virulent SSPE strains to that of the budding, non-virulent SSPE and wt strains. We report here that the M protein was not detectable in non-budding SSPE strains D.R., Biken and IP-3, and strain D.R. contained very little H protein.

Figure 1 shows the protein patterns in Vero cells infected with wt Edmonston virus and 3 cell-associated SSPE viruses, Biken, IP-3 and D.R. Uninfected Vero cells and purified Edmonston virions are also included. A protein of molecular weight 60,000 designated⁶ NP was detected in all the infected cultures and was identical in size in all strains except for the Biken strain whose NP migrated slightly slower than that of others. Although the M protein was found in Edmonston infected cells it was not detectable in cultures infected with Biken, IP-3 and D.R. Other bands seemed to be identical in these cultures.

To determine whether M protein is synthesized in Vero cells infected with cell-associated SSPE virus strains we used strain-specific immune sera, raised in rabbits, to precipitate viral proteins. Immunoprecipitation of proteins from strain Edmonston infected cells by anti-Edmonston (E), anti-Mantooth (M), anti-D.R. (D) and anti-Biken (B) sera are shown in Fig. 2a. The antisera against Edmonston and Mantooth strains precipitated proteins H, NP, A, and M. The precipitation of H and M proteins was particularly heavy, whereas that of NP was weak. Antisera against the two non-budding SSPE strains D.R. and Biken did not show a detectable precipitation of the M protein. These two sera differed in their reaction to glycoprotein H, since the anti-D.R. serum precipitated very little H protein, whereas the anti-Biken showed appreciable precipitation of this protein. The proteins precipitated from Mantooth infected cells by these immune sera were identical to those described above for the Edmonston infected cells (data not shown). Immune precipitation of viral proteins from non-budding strain D.R. infected cells by rabbit immune sera are shown in Fig. 2b. It is seen that none of these sera precipitated M protein from the D.R. cultures even though there was a heavy precipitation of protein NP. Protein A was precipitated by all the antisera although it was weaker in lanes E and M than in lanes D and B. Proteins A and F₁ were not well separated due to the compression of the gels resulting from treatment in acetone for

fluorography. The polypeptide located about molecular weight 55,000 was not identified. Figure 2c shows proteins precipitated from cells infected with non-budding SSPE strain Biken by three immune sera. The precipitation pattern is similar to that of D.R. infected cells except that precipitation of the H protein by anti-Edmonston (lane E) and anti-Biken (lane B) sera was evident. Rabbit immune sera absorbed with uninfected Vero cells did not precipitate any proteins from control cells (Fig. 2e) except a band migrating slightly behind NP. The anti-Edmonston serum was weak in antibody against A. Figure 2d shows the protein pattern of purified measles virus (Edmonston strain) run in the same gel.

These results indicate that hyperimmune sera from rabbits immunized with non-budding SSPE strains D.R. and Biken do

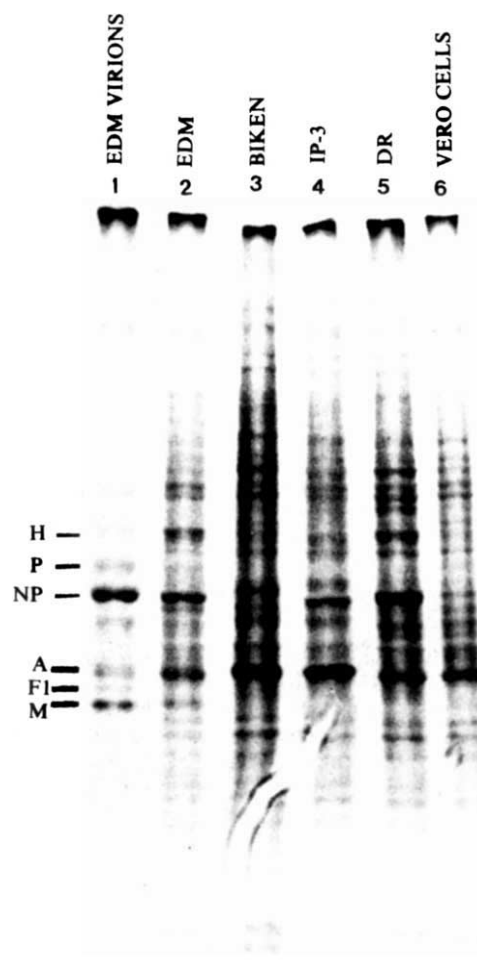


Fig. 1 Autoradiography of a slab gel containing proteins of Vero cells infected with non-budding SSPE viruses. The non-budding SSPE viruses, D.R., Biken and IP-3, were propagated by repeated trypsinization and splitting of the cell layers until the syncytia covered 50% or more of the entire monolayer, as described by Thormar *et al.*¹⁰. Cells were labelled with ³⁵S-methionine (100 μ Ci ml⁻¹, 600–1,300 Ci mmol⁻¹, Amersham), and lysed in TNE buffer (0.01 M Tris-HCl, pH 7.4, 0.1 M NaCl, 1 mM EDTA) containing 1% each of Nonidet P-40 and sodium deoxycholate and 1 mM TLCK¹¹ (*N*- α -tosyl-L-lysyl-chloromethane). Lysates were precipitated by trichloroacetic acid (10%) and the precipitate was washed twice with cold acetone. (1) Purified Edmonston virions. (2) Edmonston infected cells. (3) Biken infected cells. (4) IP-3 infected cells. (5) D.R. infected cells. (6) Uninfected cells.

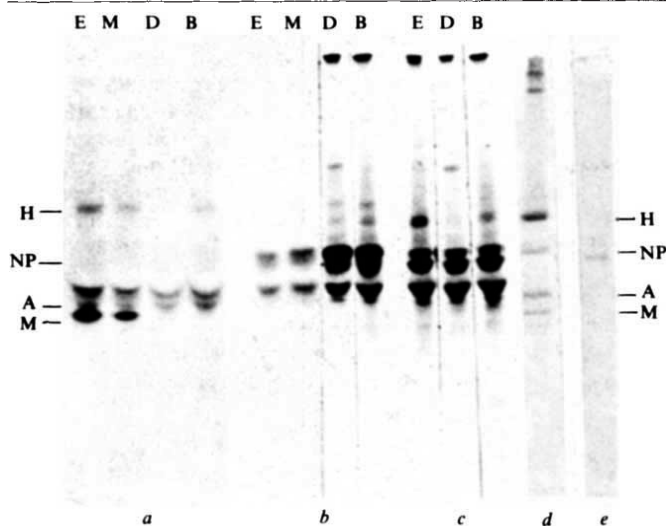


Fig. 2 Autoradiography of a slab gel containing proteins precipitated by strain specific immune sera. Vero cells were grown in 6cm Falcon plastic Petri dishes and labelled with ^{35}S -methionine as described in the legend to Fig. 1. Monolayers were rinsed twice with 3ml phosphate-buffered saline (PBS), lysed in 1ml RIPA buffer and centrifuged at 30,000 r.p.m. (Beckman 50Ti rotor) for 30 min as described by Lamb *et al.*¹¹. The supernatant was carefully recovered by pipetting and used in immunoprecipitation. To precipitate proteins, 100 μl of the extract was mixed with 20 μl of undiluted serum. The mixture was incubated at 0–4°C for 1 h and 20 μl of *Staphylococcus aureus* protein A-Sepharose CL-4B (Pharmacia)¹¹ was added to the mixture and incubation continued for 1 h. The immune complex was pelleted by centrifugation at 2,000 r.p.m. (IEC PR-6) for 20 min, washed four times with 1 ml RIPA buffer, mixed with 50 μl of sample buffer, boiled for 2 min and electrophoresed in a 5–20% gradient of polyacrylamide gel as described⁹. Fluorography was done according to the method of Bonner and Laskey¹². Strain-specific immune sera were raised in rabbits. Infected cells were prepared as described above either disrupted by sonication or lysed by 0.1% Nonidet P-40 in PBS. The cell lysate was mixed with equal volume of complete Freund's adjuvant and 1–2 ml of the mixture was injected intradermally into both hind footpads. Booster was done by injecting the same mixtures into hind legs (1 ml) 4–6 weeks later. Rabbits were bled 10 days after the booster. Sera were absorbed 2–3 times with lyophilized Vero cells until no precipitin line against Vero cells was observed in Ouchterlony test. *a*, Proteins precipitated by rabbit antisera from extracts of Vero cells infected with Edmonston strain of measles virus. E, anti-Edmonston; M, anti-Mantooth; D, anti-D.R.; B, anti-Biken. *b*, Proteins precipitated from extracts of Vero cells infected with D.R. strain of cell-associated SSPE virus. The amount of protein extract was 100 μl in lanes E and M and 200 μl in lanes D and B. The antisera were the same as used in *a*. *c*, Proteins precipitated from extract (200 μl) of cells infected with non-budding SSPE strain Biken by: E, anti-Edmonston; D, anti-D.R.; B, anti-Biken. *d*, Purified measles virus (Edmonston strain). *e*, Uninfected Vero cells incubated with anti-Edmonston serum. All the other immune sera showed similar results.

very weak. The H protein antibody was extremely strong in this serum. In contrast, the H protein antibody was very weak in the serum from SSPE patient D.R. Antibodies against the other measles virus proteins were present in the D.R. serum either comparable to or stronger than in the normal human serum. When these two human sera were run against SSPE strain D.R. the results largely confirmed those obtained with the rabbit hyperimmune serum to this strain. Precipitation of the H and M proteins was extremely weak or missing with both sera, whereas the matched D.R. serum-virus pair gave a strong precipitation of the NP, A and F₁ proteins (Fig. 3*b*). The precipitation of strain Edmonston M protein by SSPE serum may be due to antibodies remaining since the initial acute measles virus infection.

The data presented in this paper strongly indicate that the matrix or M protein is not synthesized by the non-budding SSPE virus strains D.R. and Biken in Vero cells. This conclusion is based on the absence of M protein in cells infected with these strains (Fig. 1), the failure of immune sera containing strong anti-M antibody to react with protein extracts of strain D.R. and Biken-infected cells (Fig. 2*b, c*) and the failure of strains D.R. and Biken to elicit antibodies against the M protein in immunized rabbits (Fig. 2*a*). Finally, there is no M protein precipitation in the matched D.R. serum-virus pair, in spite of the presence of anti-M antibody in the serum (Fig. 3). Absence of M protein synthesis is also indicated in Vero cells infected with non-budding strain IP-3, although the data to support this conclusion are not as strong as for strains D.R. and Biken. A reduced antibody response to M protein in sera of SSPE patients has been reported by Hall *et al.*⁷ and Wechsler *et al.*⁸ indicating a lack of activity of this

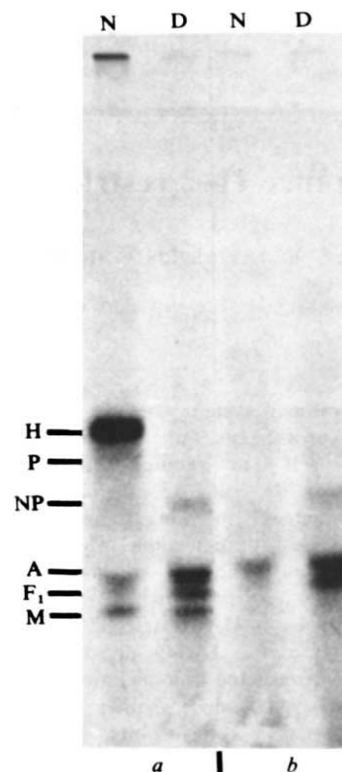


Fig. 3 Autoradiography of protein precipitates by sera from a normal person and D.R. patient. All the procedures were the same as those described in the legend to Fig. 2. *a*, Extract of Edmonston infected cells incubated with normal serum (N) or D.R. SSPE serum (D). *b*, Extract of D.R. infected cells incubated with normal serum (N) or D.R. SSPE serum (D).

not have detectable antibodies against measles virus M protein. Since a serum sample was available from patient D.R. from whom the SSPE virus strain D.R. was isolated, it was of interest to test this serum against extracts of cells infected with wt strain Edmonston and strain D.R. A normal human serum with high antibody titres against measles virus was run in the same gel as a control. Figure 3*a* shows proteins precipitated by the two human sera from Edmonston infected cells. The normal human serum (N) precipitated all the proteins of strain Edmonston although the reaction with the NP protein was

protein in SSPE patients. These data are consistent with our finding that the M protein is missing in neurovirulent, non-budding SSPE virus strains. We propose that absence of M protein is an important characteristic of these strains, not only in cell cultures but also in brains of patients. Lack of M protein synthesis and the resulting loss of ability to produce infectious virions by budding may be the critical event that turns wt measles virus into neurovirulent SSPE virus and leads to this tragic disease. We hypothesise that this is caused by a defect in or alteration of the corresponding RNA genome of measles virus. However, the possibility also exists that it is suppressed under certain conditions.

We have failed to demonstrate a significant biological or biochemical difference between budding, virion producing SSPE isolates and wt measles virus. Minor differences in the size of the M protein observed by us and also reported previously by others²⁻⁴ have not proved to be characteristic for the SSPE virus strains⁷. In view of the distinct differences between non-budding neurovirulent SSPE strains and wt measles virus, these strains rather than the budding SSPE strains may hold the clue to the unusual pathogenesis of this slow virus disease.

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Is self tolerance H-2 restricted?

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An individual's immune system must be capable of responding to a wide variety of antigens, but must not react against tissues of the individual itself. The specificity of this 'self tolerance' is determined early in life¹⁻³ and recent work has dealt with the mechanisms by which self tolerance is maintained^{4,5}. We report here a study designed to determine whether products of the major histocompatibility complex are involved in the induction of self tolerance; in particular, whether the induction of self tolerance in the mouse is H-2 restricted. H-2 restriction refers to the finding that mouse T cells generally recognize foreign antigens only when presented in association with the products of H-2 alleles⁶⁻⁸. We questioned whether T-cell precursors are made tolerant directly by antigen alone, or whether the antigen must be associated in the cell membrane with an appropriate H-2 molecule. We find that T-cell tolerance to 'self' membrane components does not seem to be H-2 restricted and discuss the possibility that this apparent lack of H-2 restriction is due to antigen processing.

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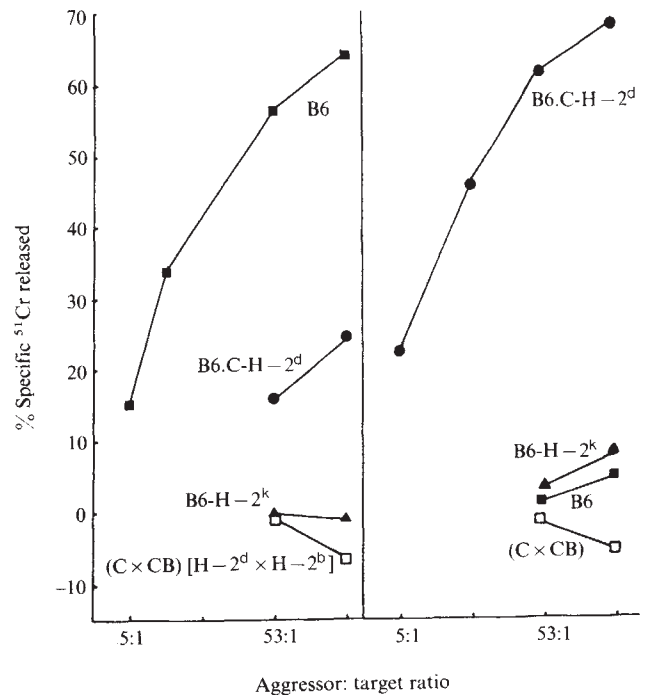


Fig. 1 Cytotoxic response of a normal (C x CB)F₁ against B6 and B6.C-H-2^d. The mouse was primed with an intraperitoneal (i.p.) injection of 10⁷ B6 spleen cells (and thus cross-primed for B6.C-H-2^d). After 9 months its spleen cells were boosted in a 5-day *in vitro* culture with mitomycin-C treated B6 (H-2^b)(a), or B6.C-H-2^d(b) stimulators and tested in a standard 4 h ⁵¹Cr-release assay for activity on 2-day concanavalin A (Con A)-induced targets as described previously¹⁵. Spontaneous release was 23% (B6), 29% (B6.C-H-2^d), 27% (C x CB) and 19% (B6-H-2^k). Effector target ratio is based on the number of spleen cells originally cultured.

We investigated the specificity of tolerance in radiation chimaeras in which donor and host differed by minor histocompatibility (H) antigens. Normal (BALB/c x BALB.B) (C x C.B) [H-2^d x H-2^b] mice respond very well to minor H antigens of the C57BL/6 (B6) strain whether the minor H antigens are associated with H-2^b (as in B6 itself) or H-2^d (as in the congenic strain B6.C-H-2^d)⁹ (Fig. 1). However (C x CB)F₁ fetal liver cells which have matured into T cells in an irradiated B6 (H-2^b) mouse would be expected to be tolerant of B6 minor H antigens associated with H-2^b. The question is: are such chimaeras tolerant of B6 minor H antigens when presented in association with H-2^d, a combination of antigens which is not expressed by any individual cell in the chimaera?

The answer depends on whether tolerance induction is H-2 restricted. If tolerance could arise by the presentation of the minor H antigens alone¹⁰ then the chimaeras should be tolerant of B6.C-H-2^d. However, if tolerance induction is an H-2 restricted event, then tolerance to B6.C-H-2^d should not occur since no cell in the chimaera carries the genes coding for this combination of antigens.

To test the chimaeras for tolerance to the combination of B6 minor H antigens plus H-2^d, we immunized them by intraperitoneal injection with B6.C-H-2^d cells. Between three weeks and two months later their spleens were removed, boosted in 5-day cultures with B6.C-H-2^d or fully allogeneic stimulators, and then assayed for cytotoxic activity on various targets. Figure 2 shows data from a representative experiment. It can be seen that the chimaeras respond well to allogeneic targets but have no activity against B6.C-H-2^d. Thus they appear to be unresponsive to B6 minor H antigens associated with H-2^d even though no cell in the chimaera is genetically capable of expressing this combination.

Table 1 Cytotoxic T-cell responses of (C × CB) → B6 chimaeras

Animal no.	Primed with:	Cultured with:	% Specific release of ⁵¹ Cr-labelled targets							
			B6.C-H-2 ^d	D1.C	Third party* (D1 × C)	C	(C × CB)	CB	B6	(C × B6)
1	B6.C-H-2 ^d	BALB.K (H-2 ^k)			44 (BALB.K)	2			6	
		B6.C-H-2 ^d	1	2						-7
		D1.C (H-2 ^d)	3	3		1				-4
2	D1.C + B6.C-H-2 ^d	B10.S (H-2 ^s)			39 (B10.S)	-2		5	3	
		B6.C-H-2 ^d	-4	-3		-2		1	5	
		D1.C (H-2 ^a)	-4	25		2		6	6	
3	D1.C + B6.C-H-2 ^d	DBA/1			65 (DBA/1)		1		3	
		B6.C-H-2 ^d	-1				1		3	
		D1.C	-1	51			0		0	
4	D1.C + B6.C-H-2 ^d	BALB.K (H-2 ^k)			40 (BALB.K)	17			2	
		D1.C	-1	75		4				-5
						-1				

(C × CB) → B6 chimaeras were primed, cultured and assayed as described in Fig. 2.

*Allogeneic, H-2 disparate stimulators and targets. The actual target is given in parentheses. Two chimaeras (not shown) had excellent killing activity against both D1.C and B6.C-H-2^d. Neither of these mice carried any (C × CB) cells, were therefore 100% B6 and recognized B6.C-H-2^d as a foreign H-2 type. Thus transient passage of (C × CB) fetal liver was not sufficient to tolerize.

Although tolerance is the most likely explanation for the lack of response to B6.C-H-2^d, an alternative suggested by the experiments of Bevan¹¹ and Zinkernagel *et al.*¹² needed to be ruled out. Their experiments demonstrated that F₁ T cells which had developed in a homozygous parental environment may respond poorly or not at all to an antigen when it is presented with the H-2 type of the unshared parent. Therefore, it could be argued that (C × CB)F₁ → B6 chimaeras would not respond to any antigen presented with H-2^d because of this host limit on restriction. However the host-limited restriction does not seem to be absolute for anti-minor responses¹¹ and can be completely overcome by appropriate immunization¹³. It is therefore possible to determine experimentally whether the lack of response to B6.C-H-2^d is due to host-limited restriction or tolerance. If appropriately immunized T cells from the (C × CB) → B6 chimaeras turn out to be capable of responding to foreign minor H antigens + H-2^d, it would suggest that their unresponsiveness to B6.C-H-2^d (self minor H antigens + H-2^d) is due to tolerance induction and not host limitation.

We thus immunized several chimaeras with D1.C (H-2^d) as well as B6.C-H-2^d cells. D1.C, an H-2^d congenic of DBA/1 (D1, H-2^a) carries at least two minor H antigens which are foreign to both B6 and (C × CB)F₁ (ref. 14). As seen in Fig. 3, the chimaeras responded against D1.C (H-2^d) and fully allogeneic targets but remained completely unresponsive to B6.C-H-2^d.

There remained the possibility that the response seen against D1.C was not an H-2 restricted anti-minor response. Several investigators have recently reported the discovery of loci (Qa, Qed-1) closely linked to H-2, whose products can elicit T-cell responses which are not H-2 restricted^{15,16}. Since the response to D1.C (H-2^d) was used to establish that the (C × CB) → B6 chimaeras could respond to minor H antigens in an H-2^d restricted fashion, it was necessary to demonstrate that this response was actually directed against D1 minor H antigens associated with H-2^d and not against any of the unrestricted Qed-1-like antigens known, or as yet undiscovered. We therefore tested the CTL against (D1 × C)F₁ targets. CTL directed against D1 minor H antigens + H-2^d should not lyse targets carrying the appropriate minor H antigens with the wrong H-2 (D1, H-2^a) or those carrying the appropriate H-2 without the minor H antigen (C, H-2^d). However, they should be active against an F₁ target which has received the appropriate H-2 from one parent and the right minor H antigens from the other (D1 × C) (H-2^a × H-2^d). If the anti-D1.C CTL are actually directed against non-H-2 restricted, Qed-1-like antigens, they should lyse D1. Figure 3 shows that (C × CB)F₁ → B6 CTL primed and boosted with

D1.C (H-2^d) do not lyse D1(H-2^a) or (C × CB) targets, but are active against both D1.C and (D1 × C)F₁ targets. We conclude that the activity is actually directed against D1 minor H antigens + H-2^d and that this is therefore an H-2^d restricted anti-minor H antigen response rather than an unrestricted anti-Qed-1-like response.

We have shown that (C × CB)F₁ T cells which have developed in a B6 environment are capable of responding to foreign minor histocompatibility antigens in association with H-2^d and that they are nevertheless completely unresponsive

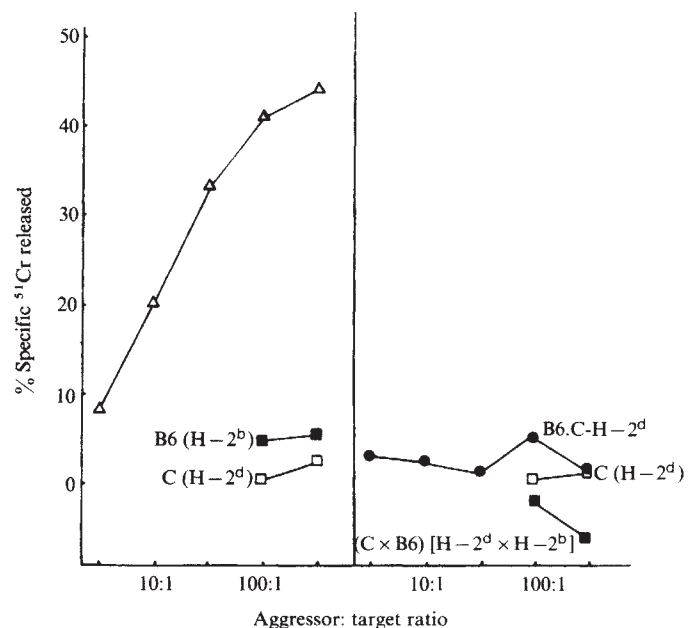


Fig. 2 Cytotoxic response of a (C × CB) → B6 radiation chimaera to B6.C-H-2^d. Chimaeras were constructed by reconstituting lethally irradiated 4 month old C57BL/6 (H-2^b) mice (925r) with 15-40 × 10⁶ 15-17 day (C × CB) (H-2^d × H-2^b) viable fetal liver cells. Two months after reconstitution some of the chimaeras were primed by i.p. injection of 10⁷ mitomycin treated B6.C-H-2^d spleen cells. Three weeks later spleen cells from a chimaera were serologically evaluated for degree of chimaerism (90% C × CB) boosted in 5-day *in vitro* cultures with mitomycin treated BALB/K (H-2^k) (a) or B6.C-H-2^d (b), stimulators, and assayed for cell mediated cytotoxicity on Con A blasts.

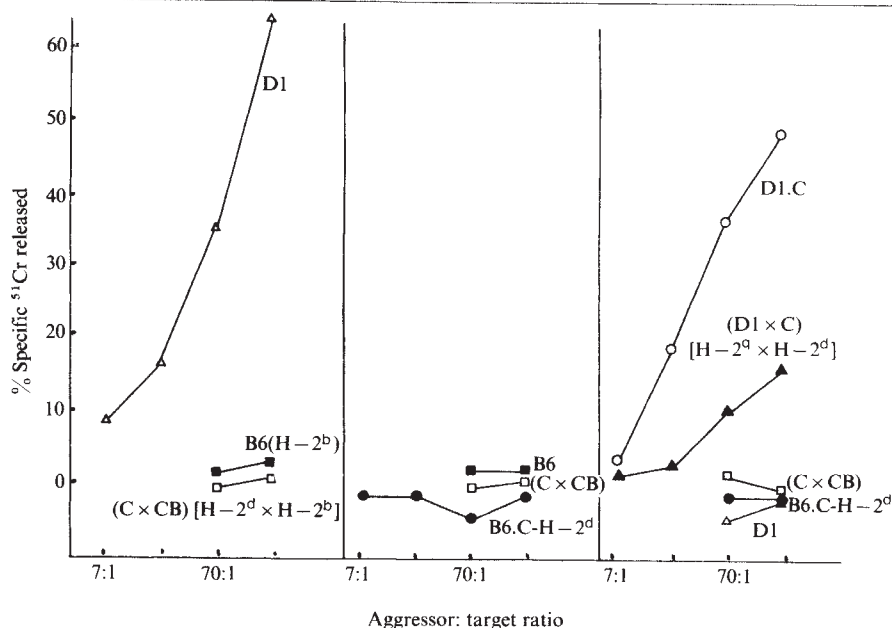


Fig. 3 Cytotoxic response of a (C x CB) → B6 radiation chimaera to D1.C (H-2^d) and B6.C-H-2^d. Two months after repopulation the chimaera was primed by i.p. injection of 10⁷ each mitomycin treated D1.C (H-2^d) and B6.C-H-2^d spleen cells. Three weeks later spleen cells from the chimaeras were serologically evaluated for the degree of chimaerism (99% C x CB), boosted in 5-day *in vitro* culture with a, D1(H-2^a); b, B6.C-H-2^d; or c, D1.C (H-2^d) stimulators and assayed for cell mediated cytotoxicity.

to B6 minor H antigens presented in the same association (Table 1). They thus seem to be tolerant of the membrane components of the chimaera in combinations which no cell in the chimaera is genetically capable of expressing. We do not yet know whether such tolerance is mediated by suppression or deletion of the T cells which are specific for B6.C-H-2^d. In either case the induction of self tolerance does not seem to be H-2 restricted.

An early hint that tolerance induction might not be restricted came from the experiments of Billingham and coworkers using H-Y antigen¹⁷ in which they rendered female mice tolerant of syngeneic male skin by a neonatal injection with H-2 incompatible male bone marrow. These females were subsequently found to be tolerant of syngeneic male skin even though the combination of self H-2 + H-Y was not found on the tolerizing marrow cells. Billingham and Silvers conducted their study to evaluate the degree of similarity between H-Y antigens of different strains of mice. Since H-2 restriction was not known in those days, its absence went unnoticed. Similarly von Boehmer *et al.*¹⁸ observed that H-2^b male → (H-2^b x H-2^k) female chimaeras did not respond to H-2^k male cells. However, this may not have reflected a state of tolerance to H-2^k male cells. Von Boehmer *et al.* have shown that the generation of killer cells specific for H-2^k male cells requires the presence of helper cells specific for H-2^b male cells. Since the H-2^b male → (H-2^b x H-2^k) female chimaeras should be tolerant of H-2^b male cells at the helper cell level, their failure to respond to H-2^k male cells may not necessarily reflect tolerance of H-2^k male cells at the killer cell level.

Our study re-emphasizes the earlier findings and shows that the unresponsiveness is most likely a reflection of a state of true tolerance.

These results lead to two possible interpretations. The first is that the induction of tolerance is not really H-2 restricted. We find this unappealing. It has been shown that the apparent non-restriction of the *in vivo* induction of T killer cells against minor antigens is actually an H-2 restricted process which appears unrestricted because of host antigen processing cells^{19,20}. We favour the view that tolerance induction is a similarly restricted event, also disguised by antigen processing. If so, what is the antigen processing cell? Does it function in the thymus or the periphery? And does it lead to a tolerant state mediated by suppression or deletion of the reactive T cell precursors?

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Inhibition of suppressor T-cell development following deoxyguanosine administration

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The expression of immunodeficiency in patients with specific purine enzyme defects indicates a crucial role of the purine salvage pathway in the acquisition and expression of normal immune function¹. One current hypothesis links the failure of normal lymphocyte development in these diseases to the accumulation of deoxynucleotide triphosphates^{2,3}. In our studies of human *in vitro* IgM responses⁴, we observed that antigen-

induced T-suppressor cell activity was abrogated in the presence of micromolar concentrations of deoxyguanosine (dGuo)⁵. In contrast, more than 1,000-fold higher resistance to dGuo was found for both non-proliferative T-helper cell activity and the differentiation and proliferation of the precursor B lymphocytes for direct haemolytic plaque forming cells (PFC)^{4,5}. To determine whether these observations could have *in vivo* relevance, we monitored the generation of murine T-suppressor cells, capable of abrogating a primary IgM response. It was found that dGuo (but not guanosine) selectively inhibited the *in vivo* development of T-suppressor cells.

Groups of 3–5 DBA/J mice (Jackson Laboratories) were immunized intraperitoneally with varying doses of the thymus-dependent, picrylated antigens ovalbumin or keyhole limpet haemocyanin (TNP-OA, TNP-KLH)⁶. After 4–6 days, direct TNP-specific PFC responses were monitored in spleen cells using fluid phase⁴ and soft agar plaque assay procedures⁶ with comparable results. In seven experiments bell-shaped antigen dose responses were observed with optimal numbers of PFC generated at 10 µg TNP-OA (Fig. 1) or 50 µg TNP-KLH. PFC responses declined from the optimal levels in an antigen dose-dependent fashion and background values were reached when ≥ 1 mg of either antigen was injected.

In man antigen dose-dependent suppression has been shown to reflect the activation, by antigen, of proliferating suppressor

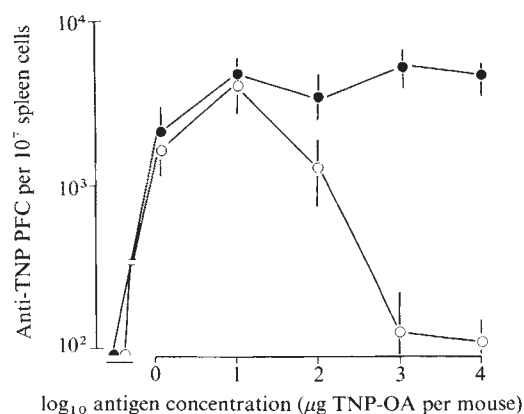


Fig. 1 Effect of dGuo on antigen dose responses of DBA/J mice to TNP-OA. Direct TNP-specific PFC were measured in Ficoll-Hypaque purified spleen cells 5 days after intraperitoneal injection of the antigen dose indicated, using picrylated sheep erythrocytes in a fluid phase haemolytic plaque assay⁵. ●, Mice receiving 1 mg per day dGuo (Sigma) throughout the immunization period. Following the injection of 1 mg dGuo serum levels of 0.2 µM were measured by HPLC for 2–4 h. ○, Pooled data from control mice, injected daily with saline or guanosine (1 mg per day, Sigma). Numbers of background PFC towards non-haptenated sheep red cells were subtracted from anti-TNP sheep red cell plaques and the results expressed as mean TNP-specific PFC ± 1 s.d. per 10⁷ spleen cells. PFC responses in animals who received no antigen (—) were below the detection threshold of the assay used (<100 PFC per 10⁷ spleen cells). One of seven similar experiments is shown.

T cells⁴. As the generation of these cells was inhibited in the presence of dGuo⁵, mice were injected with 0.1–10 mg dGuo per day throughout the immunization period. Controls received saline or guanosine. All mice survived the 4–6 day treatment period and body and organ weights (spleen, kidney, liver) were not significantly different in treated and control groups. Optimal PFC responses were not impaired in treated animals using TNP-OA (Fig. 1) or TNP-KLH (data not shown) as antigens. However, mice receiving 1 or 10 mg dGuo consistently failed to demonstrate any antigen dose-dependent

Table 1 Inhibition of suppressor T-cell development

Donor mice Treatment	TNP-OA	Anti-TNP response per 10 ⁷ recipient spleen cells	
		Anti-Thy 1.2 —	+
Control		2,580 $\pm$ 560	2,320 $\pm$ 450
Saline		2,940 $\pm$ 390	3,050 $\pm$ 310
Guanosine	10 µg	2,640 $\pm$ 330	ND
Deoxyguanosine		3,350 $\pm$ 480	3,180 $\pm$ 430
Saline		320 $\pm$ 110	2,440 $\pm$ 520
Guanosine	1 mg	470 $\pm$ 80	2,190 $\pm$ 410
Deoxyguanosine		2,670 $\pm$ 440	2,989 $\pm$ 360

Groups of four donor mice were immunized with 10 µg or 1 mg TNP-OA and received a daily i.p. injection of deoxyguanosine (1 mg), guanosine (1 mg) or saline. On day 5, spleen cells were recovered and incubated with anti-Thy 1.2 plus complement (+) or with complement alone (—). Recipient mice (3–4 per group) received 10 µg TNP-OA and 10⁷ treated (+ or —) donor spleen cells. Splenic PFC responses in recipient mice ($\bar{x} \pm 1$ s.d.) were assayed after 5 days. The monoclonal anti-Thy 1.2 reagent F7D5 was a gift from Dr P. Lake. ND, Not done.

suppression at supra-optimal antigen concentrations. In contrast, suppression was observed in all control groups, including those mice injected with 1 or 10 mg per day guanosine. When 0.1 mg or less dGuo was injected per day, PFC responses at high antigen doses were variable and approached the low control values.

To analyse the cellular requirements for high dose antigen-induced suppression of PFC responses, a cell transfer protocol was used. Mice received either a suppressive antigen dose of 1 mg TNP-OA, an optimal dose of 10 µg TNP-OA or no antigen. After 5 days, 1×10^7 spleen cells from these 'primed' mice were transferred to recipient animals which were then immunized with an optimal antigen dose. Splenic PFC responses in recipient mice were measured after 5 days (Table 1). Spleen cells from high dose antigen-primed mice suppressed the development of optimal PFC responses in recipient mice. In contrast, cells from unprimed animals or mice who had received an optimal antigen dose were unable to do so. When suppressive spleen cells from high dose antigen-primed animals were treated with anti-Thy 1.2 plus complement before their transfer to recipient mice, their suppressive activity was eliminated.

If spleen cells were used from high dose antigen-primed mice that had been treated with dGuo (1 mg per day for 5 days), T-suppressor cell activity could not be detected in these cells transfer experiments (Table 1). These data indicated that the decline in PFC responses at supra-optimal antigen doses was mediated by antigen-induced T-suppressor cells and, since recipient animals had not been treated with dGuo, that the development but not the effect of these cells was selectively abrogated by dGuo.

The biochemical mechanism(s) involved in the dGuo-mediated abrogation of suppressor cell development remains unclear. The small population sizes of antigen-specific precursors of PFC, helper and suppressor cells precluded their direct biochemical study. Susceptibility to dGuo toxicity has been linked to the ability of a cell rapidly to accumulate and maintain high levels of intracellular deoxyguanosine triphosphate³. In man this capacity is typical for immature T cells and may at least partly reflect high dGuo-kinase and low 5'-nucleotidase activity^{7–9}. Rodents studied seemed to be similar: DBA thymocytes expressed high dGuo-kinase activity (200 pmol per h per 10⁶ cells) and low 5'-nucleotidase activity (2 nmol per h per 10⁶ cells) whereas the activity profiles were inverted in splenic lymphocytes (0.07 and 16 nmol per h per 10⁶ cells, respectively). Indeed, thymocytes (but not splenocytes) rapidly accumulated and maintained high levels of

deoxyGTP when incubated in the presence of low concentrations of exogenous dGuo (data not shown).

On the basis of these findings we propose that suppressor cell precursors may resemble thymocytes with respect to key purine salvage enzymes and deoxyGTP accumulation. If this property is indeed a marker of T-cell maturation^{7,8} then the suppressor cell population monitored may originate from a pool of rather immature T lymphocytes. Alternatively susceptibility to dGuo toxicity may be a property expressed in the course of antigen-induced suppressor cell activation. The ability of dGuo to modulate selective aspects of an immune reaction in the absence of generalized toxicity may allow selective manipulation of immune responsiveness.

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Alloactivated Lyt 1⁺2⁻ T lymphoblasts bind syngeneic Ia antigens

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A large proportion of T blasts activated in the mixed lymphocyte reaction (MLR) is capable of binding plasma membrane (PM) vesicles prepared from the stimulator strain^{1,2}. The binding of vesicles is specific, and the antigens recognized are serologically detectable H-2K, D and I region products^{3,4}. T blasts binding stimulator K (and D) antigens belong to the Lyt 1⁺2⁺ subclass, whereas those binding stimulator Ia antigens are Lyt 1⁺1⁻ (ref. 5). We report here that antibodies against heavy chain V-region determinants (V_H)⁶, and furthermore, monoclonal⁷ or conventional antibodies against responder cell Ia determinants strongly inhibit the binding of radiolabelled stimulator vesicles. Anti-responder Ia inhibition is restricted to the Lyt 1⁺2⁻ subset of T blasts and correlates with a weak expression of Ia determinants on these cells. The relevant Ia determinants are not resynthesized after trypsin treatment of the blasts. In these conditions anti-V_H, but not anti-Ia reagents inhibit stimulator vesicle binding. Trypsinized T blasts can, however, passively absorb Ia material from supernatants of syngeneic, lipopolysaccharide (LPS)-activated spleen cells, and thus regain their susceptibility to vesicle-binding inhibition with anti-Ia sera. Acquisition of syngeneic Ia is also blocked by the anti-V_H reagent. We conclude that Lyt 1⁺2⁻ MLR blasts possess binding sites for both allogeneic (stimulator) and syngeneic Ia antigens.

Antigen-specific T-cell receptors probably represent immunoglobulin V-gene products, which may or may not be associated with major histocompatibility complex (MHC) antigens in the T-cell membrane⁸⁻¹². To study the possible involvement of MHC antigens in T-cell receptor function, we have attempted to inhibit the specific binding of radiolabelled stimulator PM vesicles by MLR responder T blasts using antibodies against MHC (and non-MHC) products expressed on the blasts. The representative experiment in Table 1 demonstrates that monoclonal antibodies against Ia-antigen determinants encoded by the I-A region of the responder strain MHC, strongly inhibit vesicle binding. A monoclonal antibody recognizing E/C-region products (anti-Ia 7)⁷ caused less, though significant inhibition (unpublished data). The cell population susceptible to anti-Ia inhibition was depleted by anti-Lyt 1 plus rabbit complement (RC), and enriched by anti-Lyt 2 plus RC treatment. Anti-V_H as a possible probe for the receptor¹³ also blocked vesicle binding, irrespective of the Lyt subclass of alloantigen-binding T blasts (Table 1). Very weak expression of both Ia and V_H by these cells was demonstrable by indirect immunofluorescence (data not shown). In contrast, antibodies against non-MHC antigens strongly expressed by the T blasts, that is, Thy 1 (Tables 1, 2) or Lyt 1, Lyt 2 and Ly 6 (B.E.E. *et al.*, in preparation), did not affect antigen binding. Thus, steric hindrance does not seem to be involved in the inhibition observed. The lack of inhibition with several antisera and our failure to detect Fc receptors on blasts generated from nylon-wool-purified lymph-node cells³, renders the possibility of Fc-receptor mediated blocking also unlikely. The data indicate, therefore, that Ia antigens may be located very close to the binding sites for alloantigen on Lyt 1⁺2⁻ MLR blasts.

To clarify further the role of self-Ia antigens in stimulator vesicle binding, we have tested whether the relevant Ia molecules are endogenous or are passively acquired. As shown in Table 2, vesicle binding was not blocked by anti-responder Ia antibodies after trypsin treatment and overnight incubation of the blasts. In contrast, anti-V_H was strongly inhibitory. These cells, however, regained susceptibility to anti-Ia inhibition after incubation with supernatant of LPS-activated cells, which contained soluble syngeneic Ia material. The reduction or complete loss of self-Ia after trypsin treatment, and the uptake of syngeneic Ia from supernatants of LPS blasts were also detected by indirect immunofluorescence (Table 3). Furthermore, the data in Table 3 demonstrate that the uptake of syngeneic Ia is inhibited by anti-V_H, but not by a conventional anti-Ig serum. Identical results were obtained with Ia-negative long-term MLR T-cell lines (B.E.E. *et al.*, in preparation). Thus, anti-responder Ia inhibition of alloantigen binding requires the presence of passively acquired self-Ia determinants which are closely associated with V-gene products on the surface of MLR T blasts.

In conclusion, Lyt 1⁺2⁻ (helper) T cells proliferating in MLR apparently have binding sites for both allogeneic and syngeneic Ia determinants. Binz *et al.*¹⁴ have recently shown that T-cell receptors isolated from rat MLR blasts exhibit similar antigen-binding specificity. We have demonstrated in independent experiments that the binding of both stimulator and responder Ia antigens is specific³⁻⁵ (B.E.E. *et al.*, in preparation). As the uptake of both allo- and self-Ia is blocked by anti-V_H antibody, the binding sites for both antigens may consist of V_H. However, on the basis of these data we cannot determine whether identical or distinct sites recognize allo- and self-Ia, respectively. The fact that self-Ia *per se* (that is, without anti-Ia antibody) does not interfere with the uptake of stimulator vesicles, seems to support the latter possibility. Competitive binding experiments are in progress to clarify this issue.

During these experiments, we detected, as have others^{13,15}, some inhibition of antigen binding with antibodies against responder K and D determinants. Studies on this

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Table 1 Inhibition with anti-V_H and monoclonal anti-Ia antibodies of radiolabelled PM vesicle binding by MLR blasts: Lyt phenotype of blasts susceptible to anti-Ia inhibition

MLR		Treatment	Antisera preincubation*	Binding of ¹²⁵ I-labelled SJL vesicles	
R	S			% Labelled blasts	% Inhibition of binding
A/J	SJL	NMS + RC	—	36.4	0
			NMS	34.6	5
			Anti-Thy 1.2	32.7	10
			Anti-Ia 15 + 19	15.0	59
			Anti-V _H	6.9	81
		Anti-Lyt 1.2 + RC	—	31.2	0
			Anti-Ia 15 + 19	29.4	6
			Anti-V _H	9.0	71
		Anti-Lyt 2.2 + RC	—	44.9	0
			Anti-Ia 15 + 19	11.1	75
			Anti-V _H	9.5	79

For the MLR, responder cells were nylon-wool-passaged lymph-node cells (<2% B-cell contamination), stimulator cells were 3,300 R-irradiated spleen cells³. Responder/stimulator = 1:2. Day 4 blasts were purified on Ficoll/Urovison³ (>95% Thy 1⁺, FcR⁻). Treatment of blasts with anti-Lyt sera plus rabbit complement was as described elsewhere⁵. Per cent killing of blasts was as follows: normal mouse serum (NMS) + RC: 4.1; anti-Lyt 1.2 + RC: 45.2; anti-Lyt 2.2 + RC: 35.7. For antisera preincubation, cells were incubated with antibodies (diluted 1:25) for 15 min at 0 °C, followed by 30 min at 37 °C. Radiolabelled PM vesicles were added and incubation continued for 2 h at 37 °C. For the binding studies, SJL PM vesicles prepared and labelled as described previously^{2,4} were used at a final concentration of 4.3 µg protein ml⁻¹ (0.1 µCi per µg). Binding of vesicles was detected by autoradiography². % Labelled blasts was based on scoring of 200–600 cells per group. Monoclonal anti-Thy 1.2 was from NEN. Rabbit anti-mouse V_H was purified by affinity chromatography⁶, and used at 0.15 mg protein ml⁻¹.

Table 2 Loss of vesicle binding inhibition with anti-responder Ia but not with anti-V_H antibodies after trypsin treatment of MLR blasts

MLR		Antisera incubation	Binding of ¹²⁵ I-labelled SJL vesicles by blasts preincubated with			
R	S		Medium	% Inhibition of binding	AKR supernatant	% Inhibition of binding
B10.A	SJL	—	51.6	0	53.8	0
Day 4 cells		Anti-Ia 15 + 19	50.0	3	13.9	74
after trypsin		Anti-V _H	16.3	68	22.8	58
treatment and		R anti-MIg	48.0	7	58.0	0
overnight 37 °C		Anti-Thy 1.2	46.6	10	50.0	7

MLR, antisera and vesicle binding assay were as in Table 1. Trypsin treatment of blasts was as described elsewhere¹. AKR supernatants were produced by incubating day 3 LPS-activated spleen cells (10⁷ ml⁻¹) in RPMI 1640 without serum for 6 h at 37 °C. Supernatants were recovered after centrifugation at 250g for 15 min, and 120,000g for 60 min. Soluble Ia in supernatants was demonstrated by antibody absorption. Protein concentration in the AKR supernatant used here was 50 µg ml⁻¹. Cells were incubated for 2 h at 37 °C with medium or supernatant, and washed before incubation with antisera. Rabbit anti-mouse immunoglobulin was from Behringwerke.

Table 3 Inhibition of self-Ia binding with anti-V_H antibodies

MLR		1st antiserum incubation	Ia supernatant	2nd antiserum incubation	Indirect immunofluorescence	
R	S				% Ig ⁺ cells	% Inhibition
B10.A	SJL	—	—	Anti-Ia 1,2*	13.7	—
Day 4 cells		—	AKR	Anti-D ^k †	1.7	—
after trypsin		—	AKR	Anti-Ia 1,2	40.2	0
treatment and		Anti-V _H	AKR	Anti-Ia 1,2	12.8	68
overnight, 37 °C		R anti-MIg	AKR	Anti-Ia 1,2	39.0	3

MLR, 1st antiserum incubation and Ia supernatant were as in Tables 1 and 2. The 2nd antiserum incubation was for 45 min at 0 °C. For indirect immunofluorescence studies, fluorescein isothiocyanate-labelled F(ab')₂ of sheep anti-mouse IgG (H + L) (Cappel) was used as described^{3,4}. Ig⁺ cells were seen as a weak spotted pattern of fluorescence.

* (ABY × B10.HTT) anti-A.TL; † (B10.A(2R) × C3H.SW) anti-C3H. Antisera (100 µl) were preabsorbed as follows: anti-Ia 1,2, 4 × 10⁷ SJL spleen cells; anti-D^k, 4.5 × 10⁷ SJL LPS blasts + 1.25 × 10⁸ A/J spleen cells; R anti-MIg, 6 × 10⁷ EL4 tumour cells.

phenomenon, as well as on the possible relevance of our findings for MHC restriction^{16,17} and cell-cell collaboration, are now in progress.

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Monokine-induced synthesis of serum amyloid A protein by hepatocytes

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Infection or inflammation triggers the rapid appearance in the blood of a group of proteins known as acute phase reactants¹. Serum amyloid A protein (SAA) is an acute phase protein which is believed to be the precursor for the secondary amyloid fibril protein, known previously as amyloid of unknown origin, and now as amyloid A protein (AA). The site of synthesis of SAA has been controversial, with previous evidence suggesting that AA related proteins arise in liver²⁻⁴, connective tissues⁵, polymorphonuclear neutrophil leukocytes⁶ and spleen⁷. However, in none of these systems could SAA synthesis be induced *in vitro* and the evidence rested on studies of tissues or cells arising from pre-stimulated animals. Our aim in the present studies was to prove that the liver is capable of SAA production and to identify the specific cell responsible for synthesis. The experiments demonstrate that SAA is synthesized in the liver by hepatocytes. In addition, colchicine, currently used for the treatment of amyloidosis, blocks the secretion of SAA from the hepatocyte, as has been shown for another acute phase reactant, C-reactive protein⁸.

Most of the acute phase SAA travels in serum in association with high density lipoprotein⁹, although the physiological function of this apolipoprotein is unclear. Recent evidence suggests an immunoregulatory role for SAA when added to lymphocyte cultures *in vitro*¹⁰, whereas SAA is thought to be

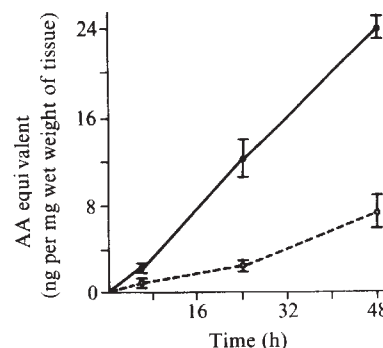


Fig. 1 Production of SAA by C57BL/10ScCR (LPS non-responder) liver organ cultures. Liver fragments (1–2 mm³) were cultured in Falcon organ culture dishes in an atmosphere of 95% O₂ and 5% CO₂. Each point represents the total SAA concentration in medium and tissue at the time of collection. Dashed line, 90% Trowell's medium and heat inactivated (56° × 30 min), 10% fetal calf serum (FCS). Solid line, 80% Trowell's medium and 10% FCS and 10% macrophage supernatant (SAASF). This was prepared from peritoneal exudate cells (PEC) collected from C3H/HeN (LPS responder) mice which had been pretreated 6 days previously with 3 ml thioglycollate broth i.p. PEC were incubated for 3 h, the non-adherent cells were removed by vigorous washing, and the adherent cells cultured with *E. coli* K235 LPS (phenol) for 24 h. It was this culture supernatant which contained SAASF.

the precursor for the secondary amyloid fibril protein^{11,12} (to be distinguished from the amyloid fibril protein of immunoglobulin origin)¹³ in certain pathological situations, especially chronic inflammation. It differs in amino acid sequence and structure from another protein known as pentagonal or 'P' component, which is found in all amyloid deposits and has been shown recently to be an acute phase serum protein in mouse, though not in other species¹⁴.

The rapid synthesis of SAA and the large amounts produced are characteristics well suited to a primary isolated cell culture system. As human SAA has the same N-terminal amino acid sequence^{15,16} and shows immunological cross-reaction with AA^{11,12}, we have used a solid-phase radioimmunoassay to detect SAA cross-reactivity to mouse AA protein¹⁷.

SAA was synthesized *in vitro* by isolated hepatocytes, cultured in the presence of an inducing factor which has been termed SAA inducer¹⁸ or SAA stimulating factor (SAASF)¹⁹. This factor is derived from lipopolysaccharide (LPS)-stimulated macrophages. The requirement for the intermediate factor has been demonstrated by using the inbred strain of mice, C57BL/10ScCR, which exhibits an abnormal response to the lipid A moiety of LPS, similar to that found in the C3H/HeJ strain²⁰. These strains have been designated LPS-non-responders, in contrast to syngeneic LPS-responder strains (C57BL/Sn and C3H/HeN, respectively). As a result of this defect, C57BL/10ScCR mice do not produce SAA when injected with lipid A or with LPS²¹ from *Escherichia coli* K235, extracted by the McIntyre method²² in hot phenol (LPS, phenol) to remove any lipid A-associated protein from the LPS²³. SAASF was initially detected in the serum of LPS responder mice 2 h after intraperitoneal (i.p.) injection of LPS, before the appearance of SAA in the serum¹⁸. This 'latent phase' serum caused SAA synthesis when administered i.p. or intravenously to LPS non-responder mice, thus demonstrating that it was not LPS carry-over which was causing SAA synthesis. SAASF activity was also detected in the supernatant culture medium of LPS-responder peritoneal exudate cells stimulated *in vitro* for 24 h with LPS. This medium, containing

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SAASF, caused SAA synthesis when injected into LPS non-responder mice *in vivo*¹⁸. In the present experiments, we have used SAASF from these supernatants to induce SAA synthesis in both liver organ cultures and hepatocyte cultures derived from LPS non-responder mice.

In initial experiments, liver fragments were cultured in plastic Petri dishes using a modification of the method of Trier²⁴. Cubes of liver tissue (1–2 mm) contained a heterogeneous group of cells, including parenchymal and non-parenchymal cells, red and white blood cells, and endothelial and connective tissue cells. Supernatants from liver fragments in culture medium supplemented with serum from healthy mice contained small, but over 24 h, increasing amounts of AA antigenic activity measured by solid-phase radioimmunoassay¹⁷. In contrast, the addition to the culture medium of latent phase serum containing SAASF increased the SAA concentration in the supernatant (from 3 to 13 ng per mg wet weight tissue at 24 h). There was little increase in AA antigenic activity in liver tissue, suggesting rapid secretion of SAA from the fragment.

Similar results were obtained when liver fragments were cultured with macrophage supernatant containing SAASF (Fig. 1). The addition of macrophage supernatant to culture medium greatly increased SAA synthesis over spontaneous production. This effect of SAASF was abolished by cycloheximide at a dose ($1 \mu\text{g ml}^{-1}$) which inhibited protein synthesis by 90%. This dose of cycloheximide also inhibited the small amount of spontaneous production of SAA in cultures without SAASF.

Although these organ culture experiments confirmed that the liver was capable of SAA synthesis, they did not define the specific cell of origin within the liver fragments. This problem was first approached by studying colchicine-pretreated, LPS-stimulated animals *in vivo*. Pretreatment of mice with

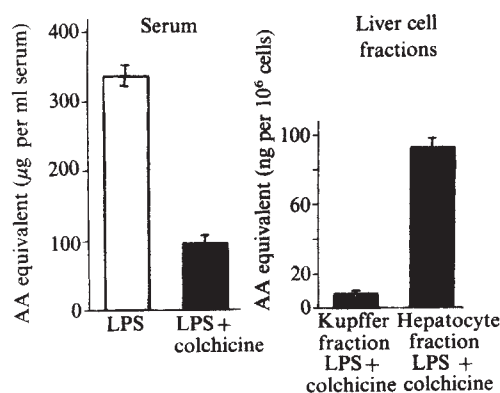


Fig. 2 Distribution of SAA in liver cell fractions from colchicine-treated LPS responder mice. Control and colchicine-pretreated C57BL/10Sn mice were injected with $1 \mu\text{g}$ LPS (K235, phenol). Liver cell fractions were prepared by collagenase perfusion of the portal vein as described by Berry and Friend²⁷. Our major modification for the smaller mouse included a reduction in pump speed to 5 ml min^{-1} for portal vein perfusion and gentle gravity sedimentation of hepatocytes as described by Jeejeebhoy²⁸ to separate parenchymal from non-parenchymal cells. The final washed cell pellet was resuspended in 90% Trowell's medium (Gibco) and 10% FCS, heat inactivated at 56°C for 30 min. Microscopy revealed a 95% pure preparation of hepatocytes. For cell culture, 4 ml of cell suspension, which contained 0.25×10^6 cells ml^{-1} , was added to a 25-cm^2 tissue culture flask and incubated at 37°C in an atmosphere of 5% CO_2 and 95% air. No hepatocytes were present in the Kupffer cell-rich suspension which had been treated with pronase. Serum and cells were assayed in the radioimmunoassay for mouse AA. □, No colchicine; ■, 0.015 mg colchicine i.p. 2 h before LPS.

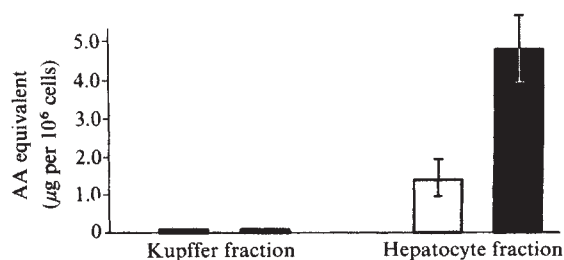


Fig. 3 SAA production by C57BL/10ScCR hepatocyte and Kupffer cell fractions. Isolated hepatocyte and Kupffer cell-rich fractions from non-responder mice were cultured with and without macrophage supernatant (see Fig. 1). Media were collected at 72 h and assayed in the radioimmunoassay for mouse AA. □, 90% Trowell's medium and 10% FCS; ■, 80% Trowell's medium and 10% FCS and 10% macrophage supernatant (SAASF).

colchicine before LPS injection resulted in a serum response which was one-third of that in mice injected with LPS alone (Fig. 2). In contrast to this blunted serum response, the colchicine-pretreated liver contained four times higher AA concentrations than LPS-injected controls (data not shown).

Subsequent experiments relied on isolation of more homogeneous liver cell populations. The technique of collagenase perfusion of the portal vein²⁵ enabled cell suspensions rich in hepatocytes or Kupffer cells to be obtained. Figure 2 shows an analysis of AA levels in isolated liver cell populations from an animal pretreated with colchicine. Hepatocyte- and Kupffer cell-rich suspensions were prepared from colchicine-pretreated, LPS-stimulated and, therefore, AA-enriched, liver. Most of the AA in liver was in the hepatocyte fraction and little in the Kupffer cell fraction.

Culture of hepatocytes and Kupffer cells more directly confirmed that SAA arises from hepatocytes (Fig. 3). There was little detectable AA in culture medium collected from the Kupffer cell-rich suspension, either with or without the addition of macrophage supernatant with SAASF. In contrast, the concentration of AA in the culture medium collected from the hepatocyte suspension was $1.5 \mu\text{g}$ per 10^6 cells and increased to $4.6 \mu\text{g}$ per 10^6 cells when supplemented with macrophage supernatant.

In conclusion, these experiments help to clarify the controversy about the origin of SAA, and provide an explanation for the prophylactic effect of colchicine in amyloidosis related to chronic inflammation. The blunted SAA response in serum seems to be secondary to a block in hepatocyte secretion of this presumed precursor of the amyloid fibril. Our data need not imply that SAA is synthesized only in the liver. Indeed, the recent finding that human SAA exists in acute phase serum in at least six different heterogeneous forms might support the contention that SAA can also be synthesized by other tissues²⁶. However, the possibility of post-translational cleavage of a common SAA precursor gene product has yet to be excluded as an explanation for this polymorphism. Of great interest is the finding that a macrophage product induces the *in vitro* synthesis of SAA, whereas the initial stimulus, LPS, does not do so directly. Thus, the many different diseases which lead to secondary amyloidosis might be expected to have in common the stimulation of macrophages which produce SAASF and thereby elevation of SAA, the putative amyloid fibril protein precursor. It seems reasonable to attribute to the resident liver macrophage, or Kupffer cell, a central role in regulating the synthesis of SAA and perhaps other acute phase proteins. Although the physiological function of SAA is unclear, we believe this to be the first report of a lipoprotein whose liver synthesis is regulated by a soluble factor derived from cells of the reticuloendothelial system.

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Genetic heterogeneity in human neuraminidase deficiency

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There is a deficiency of human α -N-acetylneuraminidase in several inherited diseases. In patients with mucopolipidosis I (refs 1, 2) and in adults with a variant form without bony abnormalities and mental retardation^{3,4}, both also classified as sialidoses⁴, it is the only deficient enzyme. In mucopolipidosis II ('I-cell' disease) neuraminidase is one of many deficient lysosomal hydrolases^{5–7} and a third manifestation combines deficiency of neuraminidase and β -galactosidase^{8,9}. We have investigated the genetic background of these various neuraminidase deficiencies by somatic cell hybridization and co-cultivation. The principal conclusions from work on mutant fibroblasts, reported here, are that at least three gene mutations are involved and that the combined β -galactosidase/neuraminidase deficiency is likely to be due to defective post-translational modification of these enzymes.

Table 1 summarizes the neuraminic acid content and the activities of β -galactosidase and neuraminidase in the different mutant fibroblasts and 4 days after fusion of each cell type with itself (parental fusion). The activity of neuraminidase was measured with N-acetylneuraminosyl-D-lactose and with 4-methylumbelliferyl- α -2-N-acetylneuraminic acid (provided by Dr J. S. O'Brien), and similar results were obtained with both substrates. Because of its simplicity and high sensitivity, we

have used principally the fluorometric assay, the results of which are reported here. As Table 1 shows, the neuraminidase activity in fibroblasts from a severely mentally retarded 11-year-old girl with the classical form of mucopolipidosis I (MLI), also classified as sialidosis 2 (ref. 4), was 1% of control values. Cells from a patient with a variant form described by Durand *et al.*³, and sometimes classified as sialidosis 1 (ref. 4), had a residual activity of 4–5%. This higher residual activity is probably responsible for the lower neuraminic acid content in these fibroblasts.

Fibroblasts from a mentally retarded adult male with myoclonus and ataxia¹⁰ and from a 2-year-old boy with Hurler-like features but no neurological abnormalities¹¹ had a profound neuraminidase deficiency in addition to a 10% residual activity of β -galactosidase (Table 1). These two patients had previously been classified as variant forms of G_{M1}-gangliosidosis¹² or β -galactosidase deficiency¹³ on the basis of complementation after fusion with fibroblasts from patients with a primary defect of β -galactosidase. Fibroblasts from both parents of the patient with infantile β -galactosidase/neuraminidase deficiency (β -gal⁻/neur⁻) had normal activity of β -galactosidase but neuraminidase activity was about half the mean control value (Table 1). This makes it unlikely that β -galactosidase deficiency is the primary defect in this condition. One of our patients with β -gal⁻/neur⁻ has recently been classified as sialidosis II (ref. 4). Because the primary defect in this condition has not yet been resolved and the syndromes included in the category sialidosis 2 are so different clinically, biochemically and even genetically, such classification seems somewhat premature.

We have studied the genetic background of the various neuraminidase deficiencies by complementation analysis after somatic cell hybridization. The results of enzyme assays 4 days after fusion of each cell strain with itself (parental fusion in Table 1) indicate that the polyethylene glycol (PEG) method of hybridization¹⁴ does not affect neuraminidase activity. The results of fusions of different mutant cell strains are summarized in the second column of Table 2. Compared with parental fusions, there was 4–15 times as much neuraminidase activity after fusion of I-cells with each of the other mutant fibroblasts. The activities after co-cultivation (third column of Table 2) were not higher than the average values for each pair of parental cells. The complementation observed after fusion of I-cells with each of the other mutant fibroblasts is most probably due to a correction of the post-translational defect in I-cell disease⁷. The rapid generation of neuraminidase activity and of other lysosomal hydrolases after fusion of I-cells with various other mutant fibroblasts¹⁵ would agree with a normalization of the processing and/or activation of preformed glycosidases.

The fusions of classical MLI $\times$ variant MLI did not result in complementation and these conditions probably represent different mutations within one gene. The same is true for the adult type and infantile type of β -gal⁻/neur⁻ deficiency. Fusions of each of the MLI strains with each of the β -gal⁻/neur⁻ deficient fibroblasts, however, resulted in a clear increase in neuraminidase activity (3–9 times the values after parental fusion). This indicates that two different gene mutations are involved in the neuraminidase deficiency of sialidosis 1 and the combined β -gal⁻/neur⁻. The restoration of neuraminidase activity after fusion of MLI cells with β -gal⁻/neur⁻ fibroblasts might result because one cell type, most probably MLI, is deficient in the structural part of the enzyme and the other is defective in a modifying enzyme or a regulatory factor. Another explanation could be that neuraminidase is made up of different subunits which must be normal for the expression of its activity.

Neuraminidase activity also increased after co-cultivation of the different types of MLI cells with each of the β -gal⁻/neur⁻ fibroblasts (compare last column Table 2 with average values of both parental strains in Table 1). This partial restoration of

Table 1 Activities of β -galactosidase and neuraminidase, and neuraminic acid content in mutant human fibroblasts and effect of cell fusion

Cell type	Total neuraminic acid (10^{-9} mol per mg)	β -Galactosidase	Neuraminidase	
			Unfused	After parental fusion
I-Cell*	117	4	0.3	0.2
Classical ML I*	71	730	1.3	0.7
Variant ML I*	40	850	3.8	3.9
Adult β -gal ⁻ /neur ⁻	60	45	1.3	1.5
Infantile β -gal ⁻ /neur ⁻	59	48	0.6	0.7
Heterozygous mother	16	574	31	—
Heterozygous father	17	627	34	—
Control fibroblasts				
Mean	22	630	82	—
Range	15–31 (n = 17)	350–1,050 (n = 36)	43–129 (n = 17)	—

Activities are expressed as nmol per h per mg protein and the values given are the means of three to six independent experiments on each mutant strain and on a large number of control fibroblasts. In all instances fibroblasts were cultured in Ham's F10 medium with 10% fetal calf serum and they were collected by trypsinization, rinsed in saline and centrifuged. Cells were disrupted by addition of bidistilled water to the pellet, and after shaking, the homogenate was used directly for biochemical analysis. Neuraminidase activity was determined with methylumbelliferyl substrate (provided by Drs T. Warner and J. S. O'Brien, see ref. 20 for synthesis). Cell homogenate (1 μ l) was incubated with 2 μ l 2 mM substrate in 0.25 M Na-acetate buffer, pH 4.3, for 1–2 h at 37 °C; the fluorescence of the liberated methylumbelliferone was measured after addition of 500 μ l 0.5 M sodium carbonate buffer, pH 10.7, at 448 nm. The activity of β -galactosidase was also measured with methylumbelliferyl substrate as before⁹. Total neuraminic acid content was measured after hydrolysis of the cells for 1 h at 80 °C in 0.1 N H₂SO₄ according to Warren²¹.

*Cultured fibroblasts from a patient with mucopolipidosis I were provided by Dr H. D. Bakker; those from a patient with a variant form (De PF in ref. 3) by Dr P. Durand, and those from a patient with I-cell disease by Dr A. Boué.

Table 2 Cell hybridization and co-cultivation of neuraminidase-deficient human fibroblasts

Combination of cell strains	Neuraminidase activity ($\times 10^{-9}$ mol per h per mg protein)	
	Hybridization	Co-cultivation
I-cell and class. ML I	6.2	0.5
I-cell and variant ML I	9.6	1.2
I-cell and adult β -gal ⁻ /neur ⁻	3.5	0.6
I-cell and infantile β -gal ⁻ /neur ⁻	5.8	0.2
Class. ML I and variant ML I	1.6	1.2
Adult β -gal ⁻ /neur ⁻ and infant. β -gal ⁻ /neur ⁻	0.8	1.0
Class. ML I and adult β -gal ⁻ /neur ⁻	9.4	3.7
Class. ML I and infant. β -gal ⁻ /neur ⁻	5.0	3.0
Variant ML I and adult β -gal ⁻ /neur ⁻	7.0	4.0
Variant ML I and infant. β -gal ⁻ /neur ⁻	6.8	4.9

Values are the means of three to six independent experiments. Hybridization was carried out with polyethylene glycol as before¹⁵ adapted for human fibroblasts in monolayer. 10^6 cells of both cell strains were mixed 4 days before fusion and cultivated in Ham's F10 medium with 10% fetal calf serum. The medium was changed after 3 days and 1 day later cells were rinsed twice with medium without serum, and after removal of the medium, hybridization was carried out as follows: 1 ml of 42% polyethylene glycol (PEG) molecular weight 1,000 (Koch-Light) in Ham's F10 with 15% DMSO was added. The mixture was rocked for 2 min, 1 ml 25% PEG in Ham's F10 was added, the mixture was rocked again and 8 ml Ham's F10 was added twice. After rocking, the medium was removed, the cells rinsed with Ham's F10 and then Ham's F10 with 10% fetal calf serum was added. After 4 days of cultivation in the same medium, the heterokaryon population was collected and analysed for neuraminidase activity using 4-methylumbelliferyl as substrate. Microscopy of stained preparations after cell fusion revealed that 70–90% of the cells contained more than one nucleus, and autoradiography after incorporation of ³H-thymidine showed that nearly all multinucleate cells were heterokaryons.

neuraminidase activity was investigated further by labelling the mutant fibroblasts with fluorescent polystyrene beads, followed by co-cultivation for 3 days, separation of the two cell populations by two-colour flow sorting (FACS II) according to Jongkind *et al.*¹⁶ and assay of neuraminidase. Table 3 shows a marked increase in neuraminidase activity in the combined β -gal⁻/neur⁻ fibroblasts whereas the ML I cells remain deficient. We could find no neuraminidase activity in the culture medium above the mutant cells and the labelling did not affect the enzyme activity. These experiments suggest the transfer of an unknown factor from ML I cells to fibroblasts with a combined β -gal⁻/neur⁻ deficiency, a factor which can increase the neuraminidase activity in the latter cells four- to sevenfold. Normal human fibroblasts also secrete this 'correction factor'. We found no evidence of an activator that could act *in vitro*, for mixing of homogenates of the two kinds of cells did not affect enzyme activity. The correction observed after hybridization (Table 2) and after co-cultivation (Table 3) may represent a normalization of the post-translational

Table 3 Co-cultivation of different neuraminidase-deficient cell strains and enzyme assays after two-colour flow sorting (FACS II)

Cell type	Neuraminidase activity ($\times 10^{-9}$ mol per h per mg protein)	
	Before	After 3 d
	co-cultivation	co-cultivation
Classical ML I	0.7	0.7
Infantile β -gal ⁻ /neur ⁻	0.8	5.3
Mixed cell population	0.7	2.9

Red polystyrene beads were added to the culture medium above classical ML I cells, left for 2 days, and cells were collected by trypsinization, rinsed and centrifuged (1,000 r.p.m. for 5–10 min). The same was done with green beads for β -gal⁻/neur⁻ fibroblasts. About 2×10^6 cells of each labelled type were seeded and co-cultivated in confluency for 3 days. The two labelled cell populations were then separated and collected with a FACS II cell sorter according to Jongkind *et al.*¹⁶ and neuraminidase activity was measured as described in Table 1. The values given are the mean of three independent experiments which gave very similar values.

processing of neuraminidase and β -galactosidase. It remains to be seen how neuraminidase is related to these processes, for neuraminidase deficiency can also occur without β -galactosidase deficiency. *N*-acetylneuraminic acid is, however, a common component of glycoproteins, certain glycosaminoglycans and gangliosides^{17,18}, and different neuraminidases have a role in the degradation of these compounds^{8,19}. A better understanding of the nature of the neuraminidase deficiency in the various mutant cells might resolve the interrelationship with β -galactosidase deficiency. Further characterization of the correction factor is in progress; its heat lability, affinity for concanavalin A and the fact that I-cells cannot provide it (co-cultivation studies in Table 2) suggest that it is a glycoprotein.

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Rapid response to selection for increased esterase activity on small populations of an apomictic clone of *Myzus persicae*

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It is generally thought that apomictic asexual reproduction produces offspring identical to the parent. In this paper we report changes that occur within a generation by selection on less than 25 offspring of an atypical strain of the aphid *Myzus persicae* starting from a single parthenogenetic mother. These changes consist of variations in the amount of a particular esterase (E_4). The amount of this enzyme has already been shown to be correlated with levels of resistance to insecticides in different strains of this aphid¹, and it has also been shown that E_4 binds and hydrolyses the activated form of the insecticide parathion². Recombination between homologous chromosomes has been eliminated as the source of variation and it appears that gene amplification might be one explanation of this phenomenon. It is conceivable that this phenomenon is associated with rapid evolutionary events such as the evolution of resistance to insecticides.

Heritable changes within a clone were first noticed when we 'pressurized' a clone of *M. persicae* (Sulzer) with organophosphate insecticide, and the surviving individuals showed increased esterase activity. When examined for

Table 1 Total esterase activity of selected aphids and the mean activities of their offspring

Generation no.	Low esterase		High esterase	
	Parental activity	Mean offspring activity	Parental activity	Mean offspring activity
1	0.34 →	0.47 ± 0.04 (11)	0.34 →	0.47 ± 0.04 (11)
2	0.33 →	0.44 ± 0.9 (6)	0.70 →	0.54 ± 0.12 (9)
3	0.27 →	0.26 ± 0.03 (8)	1.37 →	1.32 ± 0.11 (12)
4	0.16 →	0.34 ± 0.05 (10)	2.06 →	1.58 ± 0.11 (12)
5	0.21 →	0.46 ± 0.07 (13)	1.81 →	1.76 ± 0.17 (11)
6	0.27 →	0.41 ± 0.08 (11)	2.54 →	1.78 ± 0.12 (12)
7	0.17 →	0.61 ± 0.13 (9)	2.46 →	1.94 ± 0.10 (9)
8	0.38 →	0.35 ± 0.05 (7)	2.30 →	2.21 ± 0.11 (12)
9	0.21 →	0.51 ± 0.04 (10)	3.14 →	2.42 ± 0.10 (25)
10			2.84 →	2.44 ± 0.23 (15)
11			4.93 →	2.77 ± 0.18 (14)
12			3.33 →	4.26 ± 0.22 (15)
13			5.75 →	4.28 ± 0.16 (17)
14			5.32 →	4.21 ± 0.25 (14)

Recloned after 8 generations of selection

2.21 ± 0.11 (12) — No selection for 7 generations → 2.56 ± 0.42 (10)

No. of aphids selected on in parentheses (n). Units are μmol naphth-1-ol per h per mg aphid. Means given are s.e.m.

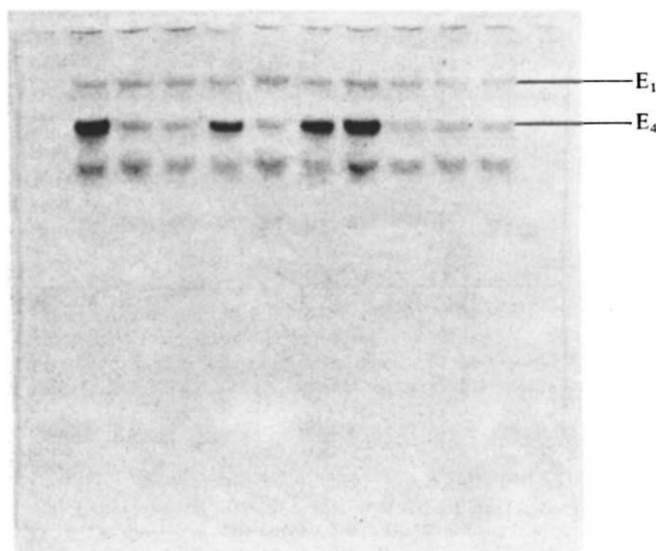


Fig. 1 Electrophoretogram of individual aphids in a culture started from a single mother of the varied clone, G_2 , to show variations in E_4 activity. These variations do not occur in all clones of this species. G_2 originates from the glasshouse of the University of Reading Department of Agriculture and Horticulture. Technique was as in ref. 2, except that a continuous borate buffer system was used, and the gel was run at 80 V for 4 h and stained for 20 min.

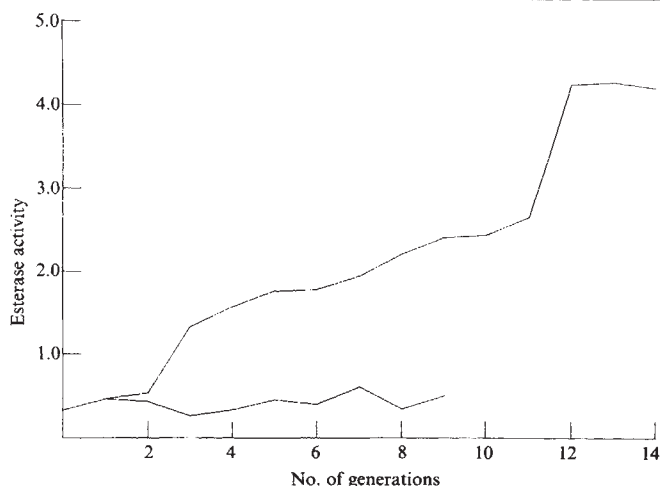


Fig. 2 Graph showing response to selection for increased and decreased esterase activity, starting from a single parthenogenetic mother. Units in μmol naphth-1-ol per h per mg aphid.

esterases by polyacrylamide gel electrophoresis², the survivors showed an increase in E_4 , the other esterases remaining constant. This could be explained by a simple inducible system, but the problem was that the offspring had high esterase as well, despite being in an insecticide-free environment. This apparently lamarckian phenomenon is explained by the fact that a single mother is able to produce offspring varied with respect to E_4 activity (Fig. 1). This latter point was found independently by Blackman³. Here we are looking in detail at the results of selection on these variations. Insecticide was not used for the selection, selection being by choice, since smaller numbers can be used this way and since the interaction of organophosphate insecticides with E_4 would complicate the results. Also one cannot select for low esterase activity with insecticide. Changes in E_4 activity were quantified by total esterase assay¹. As it is only E_4 that changes, this was a good enough index for following changes in activity at this locus.

The system of selection was as follows: A single aphid mother was taken and cultured to produce about 20 offspring. The mother was then removed and assayed for esterase activity and then, when adult, the F_1 generation were individually re-cloned to shed their offspring and were then individually assayed. The F_1 clones with the largest and smallest parental activities were selected by choice, and the rest were destroyed in alcohol. Selection for increased activity was continued for 14 generations. Selection for low esterase was continued for 9 generations. The kinetics of change are shown in Fig. 2 and Table 1.

The increase in E_4 activity over 14 generations was approximately 30 times that of the original mother. When left unselected for seven generations the mean activity of a high esterase clone had not changed noticeably but the variance in esterase activity had increased (Table 1), there being some very low and high esterase individuals in the culture. This clone exhibits genetic instability in terms of E_4 activity and resistance can be lost completely, but these reversions have not been studied in detail. Reversions to susceptibility have been reported in some instances⁴ and it seems to be the very resistant clones with a chromosomal translocation that behave in this way⁵. The clone worked on here also possesses this translocation.

What is the basis of these changes? It has long been argued that crossing-over and segregation may occur in this species⁶. However, this dramatic response to selection occurs in a clone

which is heterozygous⁷ at the E_1 locus (Fig. 3). This heterozygote has shown total stability and no homozygotes have been produced, in spite of changes at the E_4 locus. It therefore seems that the source of variation does not lie with recombination between homologous chromosomes, especially since E_1 and E_4 appear to be linked (R. L. Blackman, personal communication).

Selective gene amplification, that is the formation of multiple copies of the same gene, is one possible explanation for this variation. Selective gene amplification can occur at a very high frequency compared to normal mutations. It can be as frequent as 3×10^{-2} (R. P. Anderson, personal communication). The changes reported here can be as frequent as 3×10^{-1} (data not shown). However, it is possible that alterations in the expression of these genes are involved as well as the generation of extra gene copies.

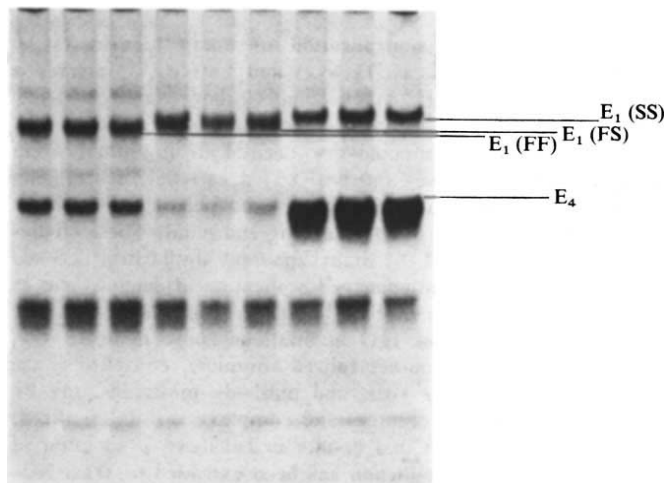


Fig. 3 Electrophoretogram showing heterozygosity of varied clone, G_2 . Samples, which are in triplicate, are of clones DDTR (fast E_1), G_2 (heterozygote) and FR6 (slow E_1). For improved resolution and separation, a 0.1 M Tris-borate-EDTA buffer was used and homogenates were run at $40 \mu\text{g}$ aphid per μl sucrose solution. Gels were run at 110 V and stained for 60 min.

There is evidence for selective gene amplification in *M. persicae*⁵, although the evidence suggests doublings of E_4 activity in different resistant clones, negating the possibility of a heterozygote between two clonal types. However, the circumstantial evidence for amplification is good. Genetic instability of increased enzyme levels is virtually diagnostic for gene amplification⁸⁻¹¹; a translocation is associated with amplification in yeasts⁸; a stepwise response to selection for increased enzyme levels is also found in cases of amplification^{10,11}; and all systems appear to be concerned with resistance or rapid evolutionary change by elevating enzyme levels.

Although the exact solution to this problem remains unknown, it is possible that the phenomenon reported here is not unique to *M. persicae* and has escaped detection in other systems because most work on selection is carried out on sexually reproducing organisms.

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An explanation for enhanced virus plaque formation in chick embryo cells

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While studying virus neutralization of many togaviruses by antisera raised in chickens, Hawkes and Lafferty^{1,2} observed a significant increase of plaque counts above controls when virus-antibody mixtures were assayed on chick embryo (CE) cell monolayers, or monolayers derived from other avian embryos. Among those antisera tested, partial neutralizing activity was observed at low dilutions and plaque enhancement at high dilutions above the neutralizing end point. These studies interested us because of the many apparent similarities between plaque enhancement and enhanced replication of dengue virus in mononuclear phagocytes. The latter has been shown to be mediated by anti-dengue IgG at dilutions above neutralization end points. When non-neutralized immune complexes are formed between dengue virus and antibody molecules, the Fc termini attach to cell receptors and complexes are internalized; this facilitates infection and results in enhanced production of virus^{3,4}. The same phenomenon has been extended to West Nile (WN) virus demonstrated on mouse macrophage cell lines by Peiris and Porterfield⁵. Here, we have examined the infection of CE cells with virus-antibody complexes of another flavivirus, Murray Valley encephalitis (MVE) virus. Our results show that a similar mechanism operates in the antibody-mediated viral plaque enhancement of MVE virus on CE cells.

MVE virus strains 96961/53 smb-7 and BH 3479/77 smb-1 were used to propagate stock virus in suckling mouse brains. At 4°C, infected mouse brains were homogenized to make a 10% (w/v) suspension in phosphate-buffered saline, pH 7.4, containing 20% heat-inactivated fetal calf serum and centrifuged at 12,000g. The supernatant fluid containing virus stock used in all experiments was stored at -70°C. Chickens were immunized with two injections of MVE virus at 3-week intervals and sera were collected at 1 and 3 weeks post-immunization. Three pools of MVE antisera were heat inactivated at 56°C and tested for neutralizing and enhancing activities in optimal conditions for enhancement as described by Hawkes¹. Our results confirmed those of Hawkes¹ and Hawkes and Lafferty²; neutralization was observed at low dilutions of antisera, followed by a two- to four-fold increase in plaque count above control values at higher dilutions. When the same virus-antibody mixtures were assayed on LLC-MK2, continuous rhesus monkey kidney cell monolayers, a greater efficiency of neutralization was observed, and there was no evidence of plaque enhancement. The same result was obtained with γ -globulins extracted from these pools of antisera as shown in Fig. 1. Viral plaque enhancement in CE cell monolayers was dependent on the Fc terminus of antibody molecules. To demonstrate this, Fc portions were cleaved from anti-MVE IgG by papain digestion⁶ and the Fab fraction purified and examined for viral enhancement. The results (Fig. 2) indicate that the Fab fraction was no longer able to mediate plaque enhancement, but retained neutralizing activity.

Viral plaque enhancement was observed when avian antisera were used and virus-antibody mixtures assayed on avian embryonic cells. Mammalian antisera from rabbits and mice

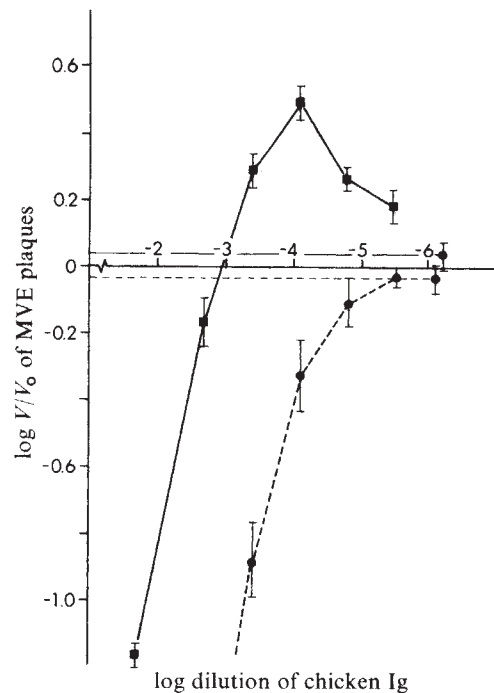


Fig. 1 Assay of mixtures of MVE-anti-MVE IgG (chicken) on CE and LLC-MK2 cell monolayers. Chicken anti-MVE γ globulin was serially diluted in Hanks' balanced salt solution containing 8% heat-inactivated normal rabbit serum, $200 \mu\text{g ml}^{-1}$ penicillin and $200 \mu\text{g ml}^{-1}$ streptomycin. Each immunoglobulin dilution was mixed with an equal volume of MVE suspension containing ~ 250 plaque-forming units per ml, then incubated at 0°C for 1.5–2 h. Virus-antibody mixtures, virus-normal immunoglobulin and virus-diluent controls were inoculated onto CE and LLC-MK2 monolayers in triplicate. After 90 min at 37°C, monolayers were covered with an agar overlay media and incubated at 37°C for 3–4 days. V and V_0 represent numbers of plaques from virus-immunoglobulin and virus-diluent mixtures, respectively. V/V_0 values are expressed as $\log_{10}$ mean $\pm$ s.e.m. from three experiments. Symbols ■ and ● represent V/V_0 values from MVE-antibody mixtures assayed on CE and LLC-MK2 monolayers, respectively. Values from normal chicken immunoglobulin controls assayed on CE cells are shown as — and from LLC-MK2 assay as ----.

when tested for their antiviral activities on CE cell monolayers demonstrated only neutralization^{1,2}. The dependence of viral plaque enhancement on a class homology between the vertebrates used to raise antibodies and the donors of tissues for cell monolayers suggested a requirement for complementarity between the Fc molecule and its receptor. A similar observation was noted by Ewald *et al.*⁷, who, using erythrocyte-antibody (EA) complexes⁸, found that sheep red blood cells (SRBC), coated with avian immunoglobulin, rosetted Fc receptor-bearing chicken lymphocytes, but SRBC coated with mammalian immunoglobulin did not; reptilian immunoglobulin was partially bound to chicken Fc receptors. These reports demonstrated complementary Fc and receptor structures within closely related phylogenetic classes. To study this phenomenon in antibody-mediated viral plaque enhancement, normal chicken γ globulins, which had been aggregated by the method of Dickler⁹, were used to treat CE monolayers at a concentration of $12.5 \mu\text{g ml}^{-1}$ for 1 h at 37°C before use of monolayers for MVE plaque assays. This treatment ablated plaque enhancement, but did not affect the formation of virus plaques or the neutralization ability of antisera. When aggregated mammalian γ globulins⁹ were

incubated with CE cells in the same manner, viral enhancement was unaffected.

We further demonstrated that plaque enhancement was associated with the presence of Fc receptors but was absent when Fc-receptor-bearing cells were removed. Antisera to SRBC raised in chickens and rabbits were used to sensitize SRBC. We tested chicken peripheral blood leukocytes (PBL), human PBL, lightly trypsinized CE and LLC-MK2 cells for EA rosette formation. Table 1 demonstrates the presence of Fc-bearing cells in chicken PBL, human PBL and CE, but their absence in LLC-MK2 cells. Again, the requirement for class homology between target cells and sensitizing antibodies for successful rosette formation was observed, confirming the observation of Ewald *et al.*⁷. Rosetted cells also exhibited immune phagocytosis. When CE-sensitized SRBC mixtures were incubated at 37°C for 90 min instead of 4°C, nearly all of the 1.7–2.0% cells exhibiting rosetting also phagocytosed sensitized SRBC. Normal SRBC and SRBC sensitized with rabbit antiserum were not phagocytosed.

Antibody-enhanced plaque formation, but not control virus plaque formation, was completely ablated by removal of CE

Table 1 EA rosette formation on chicken PBL, human PBL and trypsinized CE and LLC-MK2 cell suspensions

Cell and donor Origin of anti-SRBC	% Rosette formation			
	Chicken PBL	Human PBL	CE (suspension)	MK ₂ (suspension)
Chicken	8.02 ± 0.40	0.89 ± 0.16	2.09 ± 0.13	0
Rabbit	0.04 ± 0.03	17.27 ± 2.12	0	0

Peripheral blood mononuclear leukocytes were isolated from three human donors using methods described by Halstead and O'Rourke³ and from three chickens using the method of Lee¹⁰. Three batches of CE and LLC-MK2 monolayers were lightly trypsinized to remove cells from the glass surface. Cells were suspended in RPMI 1640 with 2% fetal calf serum at a concentration of 5×10^6 cells ml⁻¹; 0.2 ml of each cell type was mixed with an equal volume of a 0.5% suspension of normal SRBC or SRBC previously sensitized by chicken or rabbit anti-SRBC according to Ewald *et al.*⁷. Cell mixtures were incubated at 4°C for 15 min, then centrifuged at 200 g for 10 min, gently resuspended and cells examined under the microscope. A rosette was defined as a cell surrounded by three or more SRBC. At least 200 cells were examined for rosettes. Means ± s.d. from three experiments are shown.

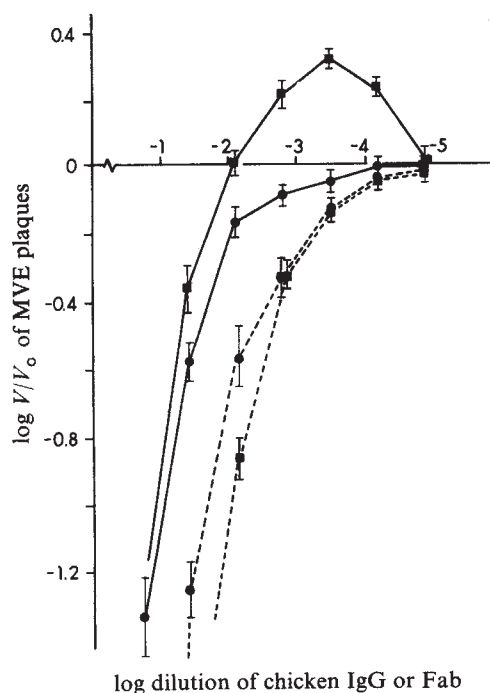


Fig. 2 Assay of MVE virus mixed with chicken anti-MVE IgG and anti-MVE Fab on CE and LLC-MK2 cell monolayers. Anti-MVE IgG at a concentration of 35 mg ml⁻¹ was treated with mercuripapain at a ratio of 1:100 (ref. 6). Digestion was allowed to proceed at 37°C for 16 h. The hydrolysates were dialysed against several changes of distilled water at 4°C, followed by further dialysis against 1.5 M NaCl and 0.01 M phosphate buffer, pH 7.0, to promote precipitation of the Fc fraction. Fab in the supernatant fraction was purified by elution through an affinity chromatography column containing Sepharose-anti-chicken Fc to remove traces of Fc and IgG. The Fab fraction was tested for purity by the Ouchterlony technique using rabbit anti-chicken Fab, rabbit anti-chicken IgG and rabbit anti-chicken Fc. Using anti-MVE IgG and anti-MVE Fab fractions, virus-antibody mixtures were made as described in Fig. 1. Mixtures were then assayed for plaques on CE and LLC-MK2 monolayers. Symbols ■—■ and ■---■ represent V/V_0 values from virus-IgG mixtures assayed on CE and LLC-MK2 monolayers, respectively; ●—● and ●---● represent V/V_0 values from virus-Fab mixtures assayed on CE and LLC-MK2 monolayers, respectively. Each point is a log₁₀ mean ± s.e.m. from three experiments.

cells exhibiting EA rosetting from CE monolayers. Fc-receptor-bearing cells were removed from trypsinized chick embryonic tissue by centrifugation of EA rosettes at 400 g for 30 min through a Ficoll-Hypaque gradient with a specific gravity of 1.078. The cells at the interface were collected, washed and plated at the usual concentration for CE monolayer formation. Compared with normals, these monolayers demonstrated a 97.5% reduction in EA-rosetted cells and a 98.5% reduction of cells capable of immune phagocytosis. The assay of MVE-antibody mixtures on these depleted monolayers resulted in neutralization only; plaque enhancement was not seen.

We conclude that normal CE monolayers are composed of approximately 2% functionally active mononuclear phagocytes. These mononuclear phagocytes engulf immune complexes and are infected. The same complexes are excluded from another cell population which does not possess Fc receptors and is infected by MVE virus without the requirement for antibody. The admixture of two distinct MVE-permissive cells is responsible for the described under-neutralization and the antibody-mediated plaque enhancement phenomenon.

As shown by Hawkes and Lafferty², immune enhancement of viral plaque formation occurs in several virus families using a broad range of embryo-derived primary tissue cultures. The demonstration of these phenomena in CE monolayers, a cell system widely used to study neutralization and the phenomenon of non-neutralizable virus fractions, requires critical review.

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Is actin in eye lens a possible factor in visual accommodation?

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Actin has been purified from various non-muscle cells and characterized by its molecular weight and ability to polymerize into filaments. Although the occurrence of this protein has been postulated in the mammalian eye lens after observation of actin-like filaments in the electron microscope^{1,2}, definite (bio)chemical proof has been provided only recently³. Amino acid analysis, peptide mapping and affinity chromatography revealed the identity of lens actin with the corresponding protein in other tissues. As the filaments could be obtained by co-isolation with highly purified lens plasma membranes, we were interested to know how the actin-containing structures were located *in situ*. In the experimental approach reported here, the indirect immunofluorescence technique (IFT)⁴ was applied to unfixed cryostat sections of lens tissue. The distribution of actin in calf, rat and pigeon lens is described, and evidence from this for the role of actin in visual accommodation discussed.

Sections, 4 µm thick, were obtained by cutting calf lenses in three mutually perpendicular directions, and incubated with specific antisera. A rabbit antiserum was raised against purified actin isolated from calf lens³. Antibodies against actin are also known to be present in the serum of patients suffering from chronic active hepatitis^{5,6}. Therefore, in addition, sera from 12 patients with clinical signs of chronic active hepatitis were selected for study. All 12 sera reacted positively with smooth muscle when tested with the IFT on rat stomach.

The sections were incubated with each of these antisera. Three projections of specific immunofluorescence could then be observed, mainly in the cortical region: (1) hexagons, the shorter sides of which showed the heaviest fluorescence (Fig. 1A); (2) equidistant and almost parallel lines of constant fluorescence (12 µm) (Fig. 1B); (3) parallel lines at constant distance (4 µm) but of interrupted fluorescence (Fig. 1C).

For an interpretation of these patterns in terms of single fibre cell structure, the model in Fig. 2A is proposed. After incubation with the fluorescent antiserum the hexagonal pattern (compare Fig. 1A) will arise from sections parallel to plane *a* (more heavily stained at the shorter sides). Conversely, strongly fluorescent lines (compare Fig. 1B) will occur in sections parallel to plane *b*. These are the lines of intersection with the short planes of the hexahedral fibres. Finally, frequently non-fluorescent lines (compare Fig. 1C) arise from the sections cut parallel to plane *c*. These lines run at relatively short distances from one another. Immunofluorescence here is mainly observed in the areas of intersection with the short planes of the hexahedral fibres. Figure 2B shows schematically the expected lines of fluorescence. From Fig. 1A–C it can be seen that actin is concentrated in specialized regions of the cells. This typical actin localization is also visualized by immuno-electron microscopy (Fig. 3). Immunoperoxidase staining reveals that actin is concentrated close to the plasma membrane and particularly at the shorter sides of the hexagon.

Figure 3B suggests that microfilaments are in close contact with the plasma membranes. A similar observation has been reported by Benedetti *et al.* in isolated plasma membranes from lens fibre cells².

Several functions have been ascribed to lenticular actin, including accommodation⁷, the process which enables the lens to change its shape so as to ensure proper diffraction of the incidental light beam. If actin has such a function, two possibilities can be envisaged. First, actin present as cytoskeletal element may enable the lens to restore its shape on accommodation. This would mean that accommodation is a passive process as far as the lens body *per se* is concerned. Second, lenticular actin may be actively involved in the process of accommodation, rendering the lens a primitive contractile organ.

Investigations on visual accommodation had shown that both extra- and intra-capsular processes are involved in lens accommodation⁸. Kleifeld *et al.*^{9–11} not only confirmed these

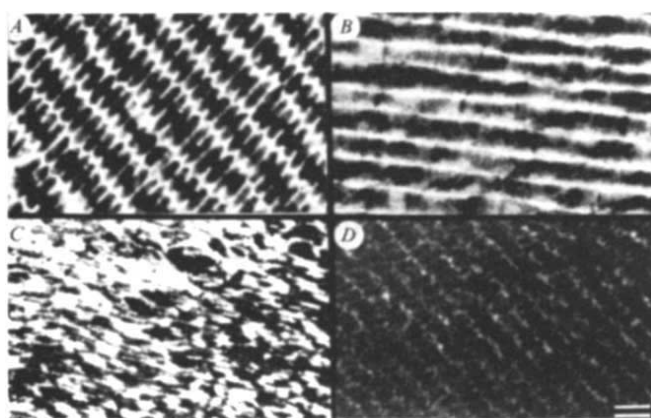


Fig. 1 Immunofluorescence of calf lens sections incubated with anti-actin serum. Unfixed cryostat sections of calf lenses (3–6-month-old animals) were used. Immediately after removal the lenses were frozen in liquid nitrogen and kept in these conditions until use. They were sectioned in three mutually perpendicular directions. The indirect immunofluorescence technique (IFT) was carried out with (1) horse anti-human immunoglobulin conjugated to fluorescein isothiocyanate (FITC) (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service lot no. PH 17-4-F8; protein concentration 14.5 mg ml⁻¹, Mol F/P ratio 2.5, final dilution 1:80); (2) horse anti-rabbit immunoglobulin conjugated to FITC-labelled polyclonal anti-human immunoglobulin or anti-rabbit immunoglobulin. The sections were studied with a Leitz microscope with incident light and dichroic mirrors. Pure calf lens actin was isolated from the cortical lens fibres as described by Kibbelaar *et al.*³. Antiserum against this preparation was produced in a rabbit by intramuscular injection of the antigen emulsified in Freund's complete adjuvant. The animal received two booster injections with the same amount of antigen. The following antisera were also used. (1) Smooth muscle antibodies (SMA) present in blood serum of patients with chronic active hepatitis⁶. Sera from 12 patients were selected. One of the sera was shown to react specifically with muscle actin⁵. (2) Sera from a healthy blood donor and from patients with myasthenia gravis that contain antibodies only against sarcoplasmic reticulum of skeletal muscle but not against smooth muscle actin, were used as controls. A, Pattern obtained from a section parallel to the hexagonal structure of the fibre cell (compare Fig. 2Aa). The shorter side of the hexagon clearly shows heavier staining. In most instances also a small part of the longer sides of the structure shows immunofluorescence. B, A continuous heavy immunofluorescence from a section cutting the fibre cell as in Fig. 2Ab. The equidistances of the fluorescent lines are larger than those in C. C, Lines obtained from sections of the fibre cell parallel to the plane in Fig. 2Ac. The discontinuous immunofluorescence indicates that only part of the fibre cell is accessible to the antibodies. D, Pattern obtained after absorption of antiserum with actin. No significant immunofluorescence is observed. Antiserum (1 ml) was diluted 10-fold in phosphate-buffered saline (PBS) at pH 7.4 and subsequently incubated with purified actin (1 mg ml⁻¹). To another 1 ml of the same antiserum 100 sections of rat smooth muscle were added. A blank incubation without tissue or actin was run simultaneously, as well as a control to which lens crystallins were added so as to discriminate between these major lens proteins and the specific actin antigen. The tubes were shaken at room temperature for 0.5 h and then incubated at 37 °C for a further 0.5 h. They were then kept overnight at 4 °C and centrifuged at 25,000g for 30 min. The supernatant was used for the IFT. All controls showed normal fluorescence. Scale bar, 20 µm.

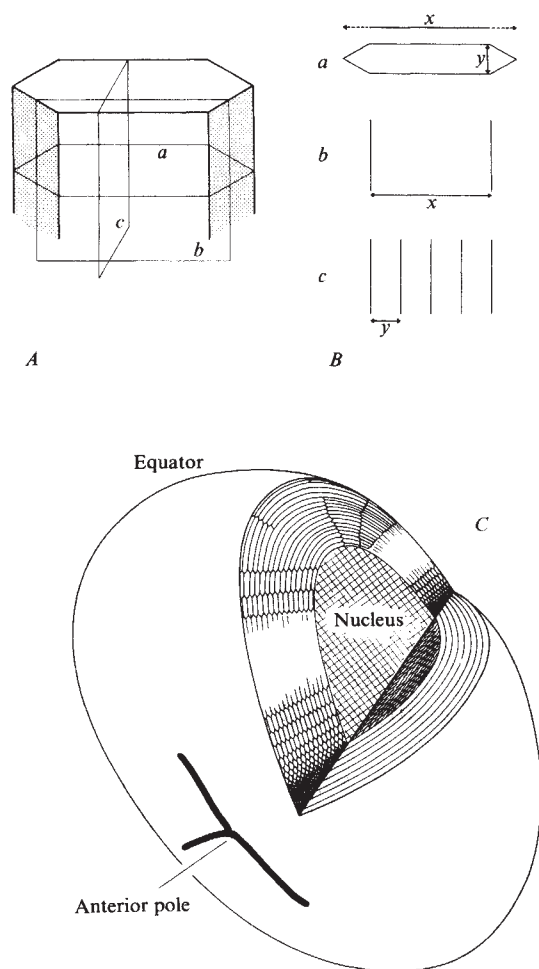


Fig. 2 A, Schematic drawing of a lens fibre with planes in three mutual perpendicular directions. Sections parallel to plane *a* show the hexagonal form of the fibre cells; sections parallel to plane *b* display the lines of intersection of the plane with the short sides of the lens fibre, and sections obtained by cutting the fibre cell parallel to plane *c* also show lines of intersections with the fibre cell, but with a smaller distance between one another than those obtained in plane *b*. B, Schematic drawing of the three main projections to be expected by sectioning the fibre cell. The distances *x* and *y* are relative to each other and do not represent real dimensions. C, Inside view of the lens illustrating the fibre cell organization. The drawing is based on a model presented by Van Heyningen¹⁸.

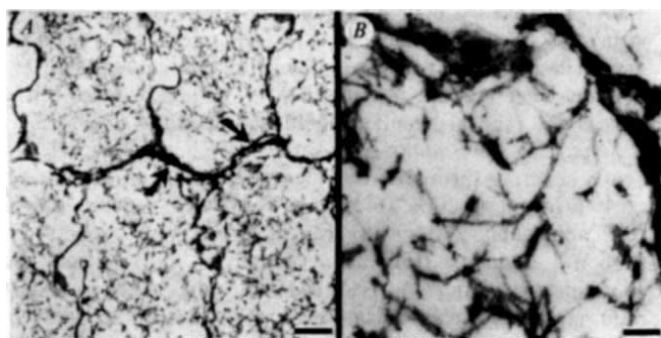


Fig. 3 Electron micrograph (immunoperoxidase staining) showing the hexagonal pattern of calf lens fibres (A). Note that the plasma membrane is covered with actin filaments (indicated by arrows, scale bar, 1 μ m). These are shown at higher magnification in B (scale bar, 0.2 μ m). The indirect immunoperoxidase staining for detection of actin was carried out as described by Poels *et al.*¹⁹ using an antiserum directed against purified lenticular actin.

ideas, but also showed that in accommodation the intra-capsular processes probably depend on active participation of the lens fibres. We wondered whether our present observations on structural features of actin *in situ* would fit these older theories. In three species (calf, rat and pigeon) which differ considerably in accommodative power and mechanism¹², striking differences in the immunofluorescence of actin were observed (Figs 4, 5).

The results of our study can be summarized as follows. In the calf, strong fluorescence occurs throughout the whole lens but is preferentially localized along the plasma membranes. In the rat, there is strong fluorescence, somewhat more intensive than in the calf, but less uniformly distributed. In the cortex, the hexagonal fluorescence pattern follows the plasma membranes (Fig. 4B). In the nucleus, however, the membrane regions appear dark whereas strong immunofluorescence fills the cytoplasm (Fig. 4C). In contrast to this latter observation, Mousa and Trevithick¹³ were unable to detect actin in rat lens nucleus. This discrepancy can presumably be explained by the electrophoretic method used by the authors, who report that 17% of the nuclear lens protein could not be dissolved in their

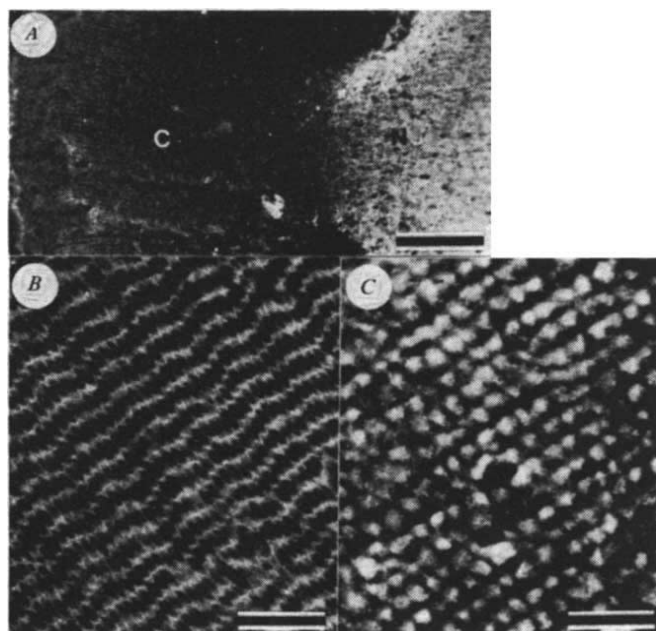


Fig. 4 Immunofluorescence of actin in rat lens sections. Sections were incubated with anti-actin serum as described in Fig. 1 legend. A, Low magnification of the rat lens showing strong fluorescence in the nucleus (N) and a weak staining in the cortical region (C) (scale bar, 100 μ m). B, Cortical region showing a preferential membrane immunofluorescence (scale bar, 20 μ m). C, Nuclear region showing strong cytoplasmic immunofluorescence (scale bar, 20 μ m).

lysis buffer. In the pigeon, there is a lower concentration of actin than in calf and rat, with almost no fluorescence in the nucleus, and weak fluorescence mainly localized in the peripheral region of the lens and virtually restricted to the cellular membranes (Fig. 5). Furthermore, electron microscopic observations show that rat and calf lenses, in contrast to pigeon lens, are relatively abundant in filamentous structures such as microfilaments and microtubules (unpublished).

Rafferty and Goossens¹⁴ hypothesized that cytoplasmic filaments in lens either structurally support its spherical shape or provide the contractile force involved in accommodation. Our data on calf and rat lens support the former assumption. The occurrence of actin filaments can indeed be correlated

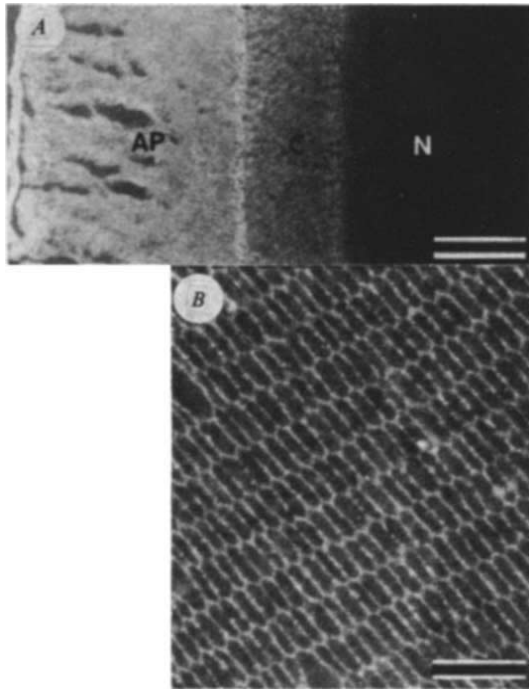


Fig. 5 Immunofluorescence of actin in pigeon lens sections. *A*, Low magnification of the pigeon lens showing moderate fluorescence in the cortical region (C) and an extremely weak fluorescence in the nucleus (N). AP, Annular pad (scale bar, 100 μ m). *B*, Detail of the cortical region showing membrane immunofluorescence (scale bar, 20 μ m).

with lenticular rigidity. On the other hand, in species in which accommodation is achieved by considerable lens deformation (such as the pigeon), structured actin seems to be scarce. However, unstructured actin may escape detection by the immunofluorescence technique. It is possible that this form of actin is actively involved in accommodation. In this respect, data in the literature concerning lenticular ATP may also be relevant. It has been suggested that the energy (ATP) required for accommodation becomes available from a special form of metabolism (called 'labour metabolism'⁸), occurring in addition to the standard metabolism of the lens. About three times higher concentrations of ATP have been demonstrated in chick and pigeon lenses than in rat and cattle^{15,16}. Moreover, in calf lens the amount of ATP differs in the various parts of the lens in that the ATP concentration in the cortex is six times higher than that in the nucleus¹⁷.

It seems, therefore, that low accommodative power is paralleled by a low ATP content as well as by the presence of structured actin, especially along the plasma membranes. Lenses with high accommodative power (as in pigeon) reveal in their flexible part only weak and diffuse immunofluorescence, suggesting a rather random distribution of actin (see Fig. 5*A*, N). Such an arrangement would permit ATP-dependent polymerization (and depolymerization), providing the lens of some species with a primitive contractile system. On the other hand, in non-accommodating animals (for example, rat) the shape of the lens is maintained by structured actin which provides the rigidity of the organ. However, this suggested molecular basis of accommodation requires further experimental substantiation.

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α -Actinin-containing branched microvilli isolated from an ascites adenocarcinoma

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Microvilli, slender projections approximately 0.1 μ m in diameter which occur on the surfaces of many cell types¹, are bounded by plasma membrane except at the site of attachment to the cell body and contain microfilament bundle cores. The presence of both microfilaments and plasma membrane suggests the use of microvilli for investigations of membrane cytoskeleton interactions. Immunofluorescence studies with anti- α -actinin² have suggested that α -actinin is concentrated at the tips of intestinal brush border microvilli and might link actin microfilaments and the plasma membrane³. However, this idea was disputed by later immunofluorescence⁴⁻⁷ and electrophoresis⁷ studies. To investigate the components and organization of microvilli from a less highly differentiated cell type, we have used an ascites sub-line (MAT-CI) of a rat mammary tumour, the 13762 mammary adenocarcinoma, whose microvilli are highly branched^{8,9}. Because such unusual structures may provide an understanding of cell-surface assemblies important in determining cell morphology, we have developed a procedure for isolating the branched microvilli and have shown that they contain significant quantities of α -actinin.

Microvilli were removed from MAT-CI cells (Fig. 1*a*) by gently shearing through a hypodermic needle, and observed by dark field microscopy (Fig. 1*b*). They were separated from cells and cellular debris by centrifugation on a self-forming Percoll gradient (Fig. 2). Microvilli are also shed spontaneously from MAT-CI cells, but the 1-3 h incubation required for significant release also yields membrane fragments and vesicles. Shearing essentially eliminates those fractions, presumably by decreasing a time-dependent breakdown of the microvilli. Microvilli can be stabilized by inclusion of 20% fetal calf serum; bovine serum albumin is ineffective.

Scanning (Fig. 1*c*) and transmission (Fig. 1*d,e*) electron microscopy of isolated microvilli showed that virtually all of them were branched; the few unbranched structures were short and were presumably fragments resulting from breakage

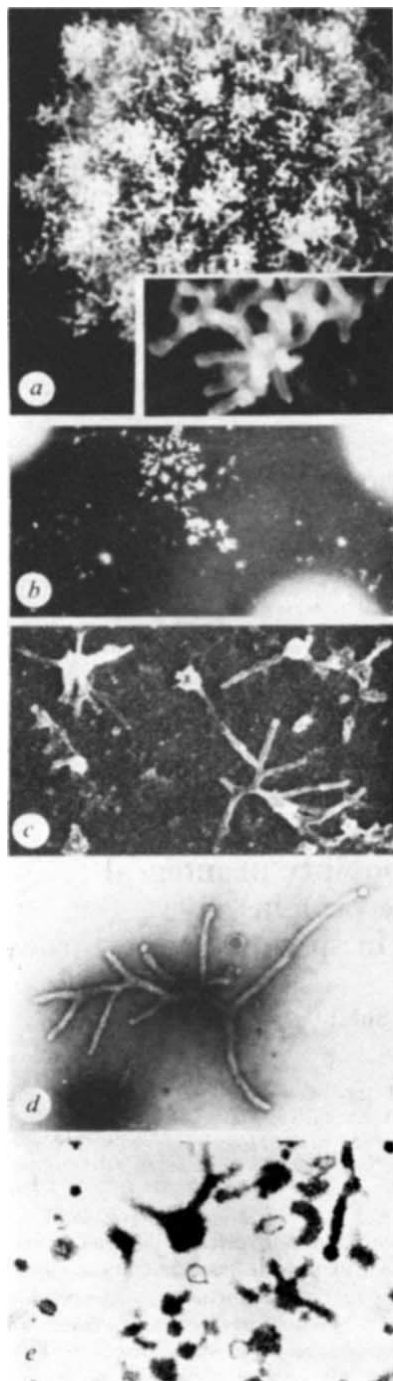


Fig. 1 Microscopic characterization of isolated microvilli prepared from MAT-Cl adenocarcinoma cells. *a*, Scanning electron micrograph of intact MAT-Cl cells ($\times 5,400$). Inset shows higher magnification of microvillus branching ($\times 20,000$); *b*, dark field microscopy of microvilli after shearing from MAT-Cl cells. The light blurs at the corners are from cells whose surfaces are out of the plane of focus; *c*, scanning electron micrograph of branched microvilli after isolation from Percoll gradient and washing ($\times 9,400$); *d*, transmission electron micrograph of branched microvilli isolated as in *c* and stained with phosphotungstic acid ($\times 20,000$); *e*, transmission electron micrograph of thin section of microvilli preparation ($\times 17,000$). For isolation of microvilli 13762 MAT-Cl cells were washed twice, suspended to $5 \times 10^7 \text{ ml}^{-1}$ in 20% fetal calf serum in phosphate-buffered saline (PBS) pH 7.4 and incubated at 37°C for 15 min. Cells were drawn gently (4–5 times) through a 14-gauge needle to shear the microvilli. The process was monitored by dark field microscopy; cell viability after shearing was greater than 95% by Trypan blue exclusion. Percoll (9:1 Percoll:10 $\times$ PBS) was added to a final concentration of 30% and the mixture centrifuged at $37,000g$ for 45 min. Microvilli were collected from the gradient with a bent needle, diluted 1:4 with 20% fetal calf serum in PBS and centrifuged at $480g$ for 10 min to pellet any contaminating cells. The supernatant was centrifuged at $48,000g$ for 60 min to obtain a soft pellet of microvilli which was transferred to a new tube and recentrifuged at $48,000g$ for 15 min. Electron microscopy was performed as previously described^{9,10}.

intact cells and isolated microvilli. The specific activity of nucleotidase was increased from 4.0 ± 0.6 to $38 \pm 5 \mu\text{mol per h per mg protein}$ (four preparations). The protein components of isolated microvilli were examined by polyacrylamide gel electrophoresis in SDS (Fig. 3). As expected, the most prominent polypeptide was a band co-migrating with actin. A second major component, with a molecular weight of about 100,000, co-migrated with turkey gizzard α -actinin. Because there has been controversy concerning the identification of α -actinin in brush border microvilli, we isolated microvilli from chicken intestine using the procedure of Bretscher and Weber⁶. Direct electrophoretic comparisons showed that the polypeptide called villin by Bretscher and Weber⁶ is different from that which co-migrates with α -actinin in our preparations. Further evidence that the latter polypeptide is actually α -actinin was obtained by 'staining' polyacrylamide gels with antibody against muscle α -actinin, using the indirect

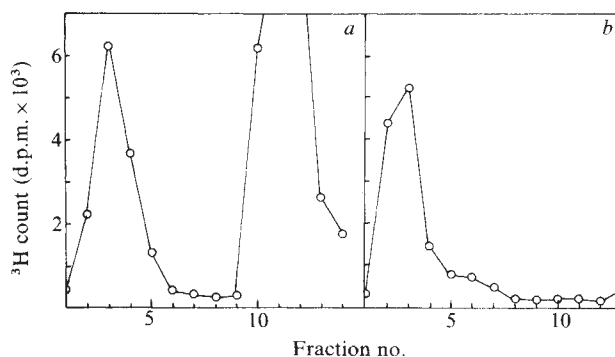


Fig. 2 *a*, Fractionation of sheared microvilli by Percoll gradient centrifugation. Cells were labelled with ^3H -glucosamine^{10,13} and used for microvilli preparations as described in Fig. 1 legend. Microvilli band at a density of 1.02 g cm^{-3} on the Percoll gradients and are well separated from cells (fractions 10–14), which are the only other significant component after shearing. *b*, Re-centrifugation of purified microvilli on a Percoll gradient gave a single peak at the same density (1.02 g cm^{-3}). The slight peak broadening probably results from some fragmentation during isolation.

during washing to remove Percoll. There were also a few spherical structures several times larger than the diameter of the microvilli, perhaps portions of the cell body removed during the shearing process or from the 'node region' at the branch points of the microvilli which became detached during purification. Branching was also clearly evident in negatively stained samples (Fig. 1*d*). Some microvilli contained a few blebs, often associated with their tips. Blebs were also observed in stained thin sections (Fig. 1*e*), which showed branch regions, longitudinal profiles and cross-section profiles. In many thin sections it was difficult to determine the orientation, that is, whether the section was cut through the microvillus core, a node region or a piece of associated cell body. Further studies will be necessary to resolve the finer anatomical details of the branched microvilli.

For additional characterization, 5'-nucleotidase, a surface component of MAT-Cl cells (refs 9, 11), was measured in

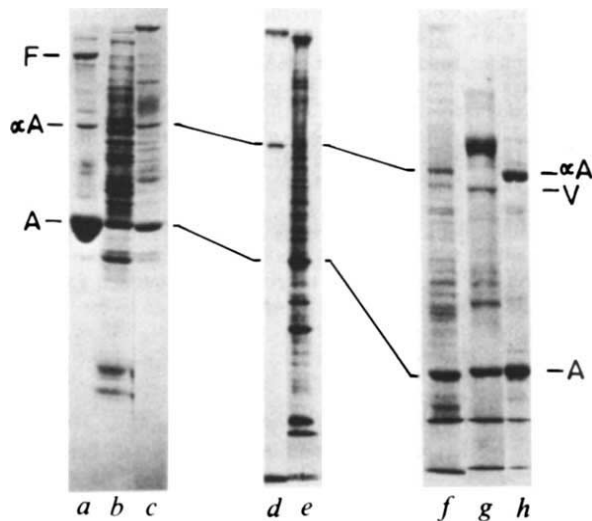


Fig. 3 Polypeptide components of isolated microvilli fractionated by polyacrylamide gel electrophoresis in SDS. Gel electrophoresis was performed as previously described^{10,13}. The first panel (a-c, 4.5–15% acrylamide gradient, Coomassie blue stain) shows a microvillus preparation (c) compared with standards (a, A, actin; α-A, α-actinin; F, filamin¹⁵) and intact cells (b). Note the concentration of actin and α-actinin in the microvilli compared with those of other cellular proteins. The second panel (d and e) shows the identification of α-actinin in a gel of the MAT-Cl microvilli by antibody 'staining' using anti-α-actinin followed by ¹²⁵I-labelled anti-IgG^{12,16}. d is an autoradiogram of the antibody-labelled gel, and e is the photograph of the corresponding gel stained with Coomassie blue (gel composition 10% acrylamide, 0.13% bisacrylamide). The stains at the top and bottom of the gel are due to labelled IgG absorbed to the gel interface rather than specific staining. The antibody 'staining' was performed using an antibody preparation described and characterized previously^{12,16,17}. The third panel (f-h, 7% acrylamide, Coomassie blue stain) shows MAT-Cl microvilli (f) compared with chicken intestine brush border microvilli prepared according to the method of Bretscher and Weber⁶ (g) and a crude preparation of turkey gizzard α-actinin (α-A in h), which still contains a substantial amount of actin. The polypeptide of molecular weight 95,000, termed villin, was identified by comparison with published results of Bretscher and Weber⁶ and is marked V. Note the absence of a band co-migrating with α-actinin in the brush border microvilli and the absence of a band co-migrating with villin in the MAT-Cl microvilli. There is a band from MAT-Cl microvilli which migrates slightly more slowly than villin, but its identity is unknown.

technique with ¹²⁵I-labelled anti-IgG (ref. 12). Only one band was observed, corresponding to the migration position of α-actinin (Fig. 3), indicating that α-actinin is a major component of our preparation of isolated microvilli.

The quantity of α-actinin present in the microvilli suggests that it has a structural role. There is no evidence to support its proposed role as a membrane-cytoskeleton linking agent, but its localization in the branched microvilli by immunofluorescence or immune electron microscopy should elucidate this question. Another question is why do MAT-Cl cells have such elaborate surface structures. As this sub-line is highly malignant and xenotransplantable^{10,13} (that is, transplantable across the species histocompatibility barrier into mice), it is tempting to speculate that the complex surface is a protective device, for example, aiding the cells in escaping immune surveillance¹⁴.

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Lateral mobility of integral membrane proteins is increased in spherocytic erythrocytes

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Alterations of glycoprotein distribution and lateral mobility in cell membranes can provide transmembrane signals for several membrane-related phenomena^{1–3}. Control of the transmembranous events has been ascribed to interaction between submembranous protein matrices (or 'cytoskeletons') and membrane glycoproteins^{4–7}. A consequence of such interaction would be differential inhibition of protein lateral diffusion in biological membranes. Measurements of the lateral diffusion coefficients of membrane proteins, in fact, have generally yielded values^{8–12} much less than were predicted for unhindered diffusion in a fluid bilayer^{13,14}. The mouse spherocytic erythrocyte, which lacks the major components of the normal erythrocyte membrane matrix¹⁵ (composed of spectrin, actin, bands 4.1 and 4.9 (ref. 16), in the nomenclature of Fairbanks *et al.*¹⁷), provides a unique system for a direct evaluation of the effect of the matrix on protein lateral mobility. After using a modification of the technique of fluorescence redistribution after photobleaching (FRAP)¹⁸, we report here that membrane proteins diffuse about 50 times faster in spherocytic than in normal mouse erythrocytes.

Integral membrane proteins were labelled with fluorescent molecules by two methods, direct labelling and fluorescent concanavalin A (Con A) binding. Intact cells were incubated

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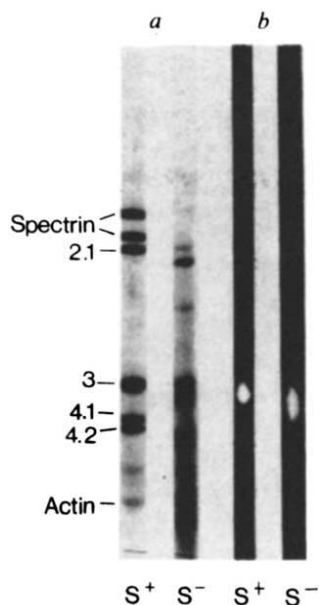


Fig. 1 SDS polyacrylamide gels (5.6% acrylamide) of DTAF labelled from S^+ and S^- erythrocytes. In *a*, the gels were stained with Coomassie blue (30 μ g of protein per gel). In *b*, unstained gels were excited with laser light at 488 nm and photographed with a 510-nm barrier filter (60 μ g of protein per gel). This pattern of fluorescence did not change when acrylamide in the gel was decreased to 3.25%, which would enable resolution of glycophorin from band 3. Cells were obtained from either S^+ or S^- (*sph/sph*) WBB6F₁ mice (males) by retro-orbital puncture with 2-mm internal diameter heparinized capillary tubes. Samples were washed three times with phosphate-buffered saline (PBS, 140 mM NaCl, 10 mM PO₄, pH 7.5) to remove plasma and the bulk of leukocytes and platelets. Packed washed cells were suspended in 2 volumes of PBS and mixed with 3 volumes of 1 mg ml⁻¹ DTAF in 0.2 M sodium borate (pH 10) on ice. After 15 min, cells were washed four times with PBS. Membranes were prepared by lysis of cells 1:100 in 5 mM NaH₂PO₄ (pH 7.4) and centrifugation (40,000 g for 10 min). This procedure was repeated once and the membrane pellet was solubilized for SDS gel electrophoresis by the procedure of Fairbanks *et al.*¹⁷.

with the fluorophore, dichlorotriazinylaminofluorescein (DTAF), at pH 10, so that a single protein was labelled in normal erythrocytes (S^+) and a broad protein band of similar molecular weight was labelled in the spherocytic erythrocytes (S^-) (Fig. 1). None of the major peripheral proteins on the cytoplasmic side of the membrane was labelled. Extraction of the labelled cells with 0.2 mM PO₄ (pH 8.0) or 0.6 M KI at 37°C for 10 min followed by centrifugation resulted in a cell pellet containing 85% of the original fluorescence. The amount of total label that electrophoresed with lipids was less than 10% in the normal mouse cells and 25% or less in the S^- mouse cells. Most of the label was found on the protein, band 3, or a variant of it in the spherocytic cells.

When labelled cells were scanned with a low-intensity focused laser beam (beam diameter $\sim 1 \mu$ m), fluorescence was distributed as shown in Fig. 2. After two or three such scans a bleach was introduced at the edge of the cell, producing an asymmetrical distribution of fluorescence. Further scans show that the fluorescence redistributed as a function of time, the rate of redistribution being much greater in S^- membranes than in S^+ membranes (Fig. 2). The diffusion coefficients in Table 1 were derived from the scans by a new normal mode analysis of diffusion on a spherical surface, described in detail elsewhere¹⁹.

Additional evidence that the fast lateral mobility in S^- cells was due to the diffusion of glycoproteins and not lipid was

obtained by cross-linking membrane glycoproteins with the tetravalent lectin, Con A. Con A at a concentration of 50 μ g ml⁻¹ or greater completely immobilized fluorescent components on spherocytic cells, but low concentrations did not cause immobilization. Accordingly, we labelled S^- cells with low concentrations of rhodaminated Con A to provide another fluorescent label for FRAP. The mobility of the Con A bound to glycoproteins was as great as 1.2×10^{-9} cm² s⁻¹ or nearly that of the components labelled directly with DTAF (Table 1). Higher concentrations of rhodaminated Con A which caused extensive cross-linking of membrane glycoproteins resulted in Con A immobility.

Previous studies have suggested that the high-intensity irradiation during the bleach may affect the diffusion of protein by inducing oxidative inter-protein cross-links, especially in the presence of spectrin²⁰. Accordingly, all measurements on S^+ cell membranes were made in the presence of 5 mM glutathione, which prevents or rapidly reverses photo-induced cross-links in erythrocytes²⁰. The diffusion coefficients thus obtained are in close agreement with values obtained for human erythrocytes in which redistribution of labelled proteins was observed after polyethylene glycol-induced fusion, without photobleaching¹².

Spherocytic erythrocytes are short-lived, and so the circulating blood cells must be predominantly reticulocytes (>90%)¹⁵. Geiduschek and Singer²¹ have suggested that glycoproteins in reticulocyte membranes are more mobile than in adult erythrocyte membranes. To rule out the possibility that the difference in mobility between S^+ and S^- erythrocytes was due to the presence of reticulocytes, we labelled S^+ reticulocytes with DTAF and found a diffusion coefficient of 1.3×10^{-10} cm² s⁻¹—3 times faster than that for glycoproteins in the normal mouse erythrocyte membrane, but nearly 20 times slower than in the spherocytic mouse erythrocyte membrane. These results suggest a difference in the

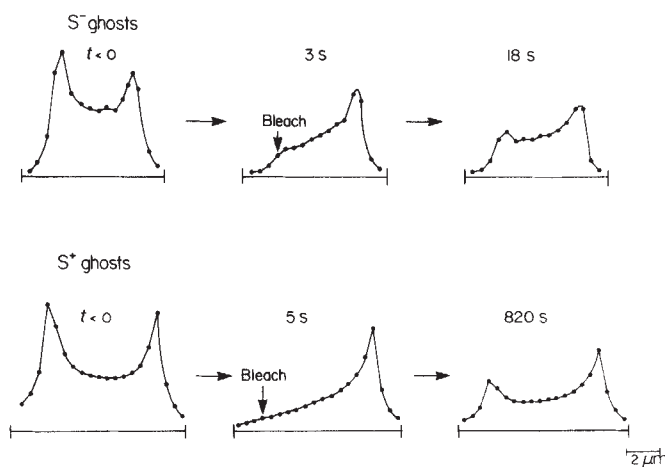


Fig. 2 Scans of the fluorescence distribution on DTAF-labelled S^- and S^+ membranes before and after irradiation of the leading edge of the cell with a bleaching pulse for 35 ms (0.1 mJ). The apparatus used here has been described elsewhere¹⁸. Single scans are collected in 1 s by scanning a focused laser beam (approximately 1 μ m in diameter) of 0.1 μ W at 4,765 Å across the membranes. Membranes of labelled S^- erythrocytes were prepared by allowing spontaneous lysis to occur on the slide. Over 60% of the cells lysed within 10 min at 24°C. Membranes of labelled S^+ erythrocytes were prepared by lysis (1:100 in 5 mM NaH₂PO₄, pH 7.5), centrifugation (40,000 g for 10 min) and resuspension in PBS with 5 mM reduced glutathione (pH 7.5). Similar diffusion values were obtained with membranes from S^+ which had spontaneously lysed during the slide preparation. Slides and coverslips were preincubated in 0.1 M NaH₂PO₄ (pH 7.5) and washed with distilled water before use. Samples on the slides were sealed with paraffin to prevent evaporation.

Table 1 Diffusion coefficients of erythrocyte and reticulocyte membranes

Membranes	Diffusion coefficient (s.d.) ($10^{-11} \text{ cm}^2 \text{ s}^{-1}$)
S^- erythrocytes	250 ± 60 (9)
S^+ reticulocytes	13 ± 4 (5)
S^+ erythrocytes	4.5 ± 0.8 (4)

Diffusion coefficients, D , were determined in a normal-mode analysis¹⁹ from experimental estimates of $\mu_1(t)$, the normalized first moment of the concentration distribution $c(x, t)$:

$$\mu_1(t) = \frac{\int_{-1}^1 xc(x, t)dx}{\int_{-1}^1 c(x, t)dx} \propto \exp(-2Dt/r^2)$$

where x is the cosine of equatorial angle θ on the surface of the spherical cell of radius r . In most cases, the data could fit to a single exponential within experimental error. The values of D reported were measured at 24°C . The number of measurements made is in parentheses. S^- and S^+ erythrocytes were prepared as in Fig. 2. For S^+ reticulocytes, reticulocytes were isolated from a mouse which had been bled (0.6–1.0 ml per day) for 3 days. Forty hours after the last bleeding a blood sample was obtained as in Fig. 1 and centrifuged at 15,000g for 2 min. The upper 25% of the packed cells was drawn off, washed and labelled as in Fig. 1. Reticulocyte membranes were obtained by the procedure described in Fig. 2 legend for S^+ membranes. For these measurements only membranes with a definite reticulum were chosen.

arrangement of reticulocyte matrices. Indeed, electron micrographs by Geiduschek and Singer document spectrin-free domains²¹.

As Fig. 1 shows, S^- cell membranes differ from S^+ cell membranes in several protein components, having less spectrin, spectrin-binding protein²² (band 2.1) and matrix-associated protein¹⁶ (band 4.1) and considerably more polypeptides in the low molecular weight region of the gel. There may be other differences, but additional evidence supports our contention that interactions between the membrane and the matrix control lateral mobility. Fowler and Bennett²³, using a procedure designed to weaken the association of spectrin with the membrane, observed a two-fold increase over controls in the lateral mobility of band 3. We have demonstrated that compounds normally found in erythrocytes, which specifically decrease interactions between spectrin, actin and band 4.1, increase the lateral mobility of membrane proteins¹². Thus, decreasing interactions between the matrix and the membrane, or between matrix components, increase membrane mobility. Conversely, in preliminary experiments, the addition of spectrin to S^- cell membranes inhibited glycoprotein mobility (M.P.S., M.S. and D.E.K., unpublished).

The presence of a membrane matrix could control protein mobility in either of two ways: by a direct mechanism involving attachment of mobile glycoproteins to cytoskeletal elements^{6,7}, or by an indirect mechanism analogous to restricted diffusion through a polymer network^{6,24}. The former mechanism would be expected to slow down protein rotational as well as translational diffusion; the latter would not. It has been demonstrated that the presence of spectrin does not affect the rotational diffusion of band 3 (ref. 25). It thus seems that in conjunction with our results an indirect control must be postulated for the spectrin, actin, bands 4.1 and 4.9 matrix inhibition of lateral mobility. We suggest that matrix control mechanisms such as this are also active in other eukaryotic cells.

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The conformation of subcomponent C1q of the first component of human complement

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The complement system in the blood of vertebrates^{1,2} has a vital role in the defence of the body against invading foreign material. Although it can be triggered before immunity has developed, it is usually activated following an initial intervention by antibodies, and about 20 serum proteins are involved. Some of these act as controlling inhibitors while others form nine components which, if following the classical activation pathway, all take part in bringing about membrane damage and cell lysis. The first component C1 consists of three subcomponents, C1q, C1r and C1s. C1q binds to antibody–antigen complexes as the first stage of fixation³ and as it shows no enzymatic activity by itself, conformational changes on binding have been proposed as the driving mechanism of the subsequent reactions. Since structural investigation by electron microscopy^{4,7} leaves doubt as to whether unnatural constraints were applied in the preparation and treatment of specimens, techniques such as X-ray or neutron small-angle scattering, which investigate the equilibrium state in dilute solution, provide valuable complementary evidence for both its findings and those of other physicochemical methods⁸. We have already carried out neutron work on the conformation of immunoglobulin and the influence of hapten binding (to be published) and are now studying the conformation of C1q as a preliminary to investigating the composite structures resulting from their mutual interaction. As evidence of the structure of this molecule has accumulated, the question has arisen of whether it maintains a compact closed conformation in its native state or a more open one in readiness for interaction with other proteins. The neutron small angle scattering curve is sensitive to conformational changes and although our measurements are not yet complete, model calculations suggest that the structural arrangement is indeed open.

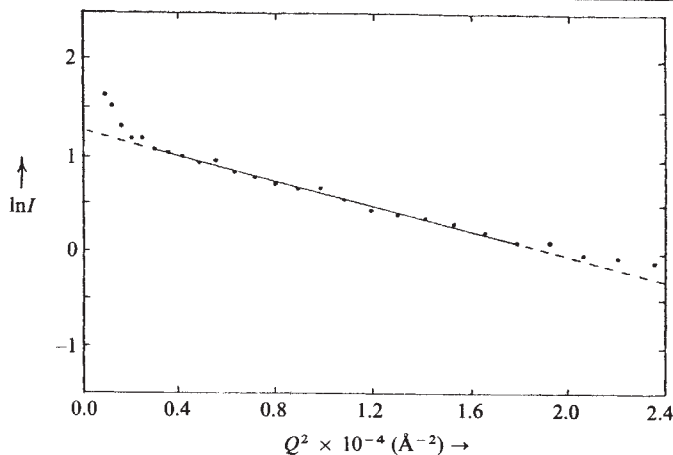


Fig. 1 Example of the $\ln I$ against Q^2 scattering curves obtained for C1q. I , corrected neutron counts on a relative scale; Q , scattering vector. The specimen solutions used were of pH 7.4 and, besides protein, contained 0.15 M sodium chloride, 0.01 M sodium phosphate and 0.01 M EDTA. They were held in high quality boron-free silica spectroscopic cells whose internal thickness was either 2 mm or, for the more highly absorbing hydrogenous sample, 1 mm. Data were collected at a specimen-detector distance of 10.5 m and a neutron wavelength of 12.1 Å ($\Delta\lambda/\lambda \approx 8\%$). Radial averaging gave intensities at annular increments of 1 cm ($\Delta(2\theta) \approx 0.03^\circ$). Control runs of cell, solvent and buffer only were subtracted with allowance for change of transmission and the results scaled according to a water sample to correct for instrumental effects. The fit to the Guinier law for the 100% exchanged C1q sample illustrated gives an apparent radius of gyration R of 140 ± 2 Å over the range $0.75 \leq QR \leq 1.85$ with a statistical correlation coefficient of 0.996. This is close to 1 (the value for a perfect straight-line relationship) and is maintained for sets of points within the range, as is the value of R . There is therefore little evidence for the presence of aggregated particles. The deviation of the first few points in the spectrum is probably due to the proximity of the detector elements to the beam stop. The intensity measurements can only be put on an absolute scale at present by reference to spectra which are less statistically reliable, but doing so allows determination of the molecular weight of C1q as $370,000 \pm 50,000$. The accepted value from sedimentation equilibrium studies is 409,600 (ref. 9).

Human C1q was prepared as described previously⁹ and made up in aqueous solutions to a concentration of 3 mg ml^{-1} . To provide a range of scattering contrast between the protein molecule and its surrounding medium¹⁰, the solvent was 0%, 85% and 100% deuterated and, except in the case of the completely hydrogenous solution, each sample of C1q was pre-dialysed against its intended solvent to allow appropriate replacement of the exchangeable hydrogen of the protein by deuterium. Neutron small-angle scattering spectra were recorded using the multidetector D11 (refs 11, 12) at the Institut Laue-Langevin, Grenoble.

The small-angle scattering of randomly orientated identical particles in solution can be represented by the gaussian intensity curve¹³

$$I(Q) = I(0) \exp -\frac{1}{3} R_g^2 Q^2 \quad (1)$$

where $Q (=4\pi \sin \theta/\lambda)$ is the scattering vector associated with a scattering angle of 2θ at wavelength λ . Within the validity of this expression ($QR_g \leq 1-1.5$) the relationship between $\ln I$ and Q^2 is linear with a slope directly related to the radius of gyration R_g of each particle. Least squares fitting of the most highly correlated range of points in the plots obtained for C1q (see Fig. 1) gave apparent radii of gyration for each contrast

and the corresponding relative value of the intercept $I(0)$. After division by the measured sample transmissions, the linear variation of $I(0)^{\frac{1}{3}}$ allowed determination of the percentage deuteration at which the mean scattering density of the protein ρ is identical to that of the solvent ρ_s .

The match point found at $41.7\% \pm 0.2\%$ corresponds to a scattering density of $2.33 \times 10^{10} \text{ cm}^{-2}$. As the total molecular weight of human C1q is 409,600 and the detailed composition of the 92% amino acids and 8% carbohydrate is known⁹, the sum of the scattering lengths of the molecule can be calculated for any proportion of exchanged hydrogen atoms. The dry volume V was therefore deduced to be $530,000 \pm 4,000 \text{ Å}^3$ and the mass density $1.28 \pm 0.01 \text{ g cm}^{-3}$, in close agreement with the result of about 1.3 g cm^{-3} expected for a protein. As the scattering density in a particle is not in general homogeneous, the observed radius of gyration R is dependent on the contrast $\bar{\rho} (= \rho - \rho_s)$ and hence¹⁴

$$R^2 = R_0^2 + \frac{\alpha}{\bar{\rho}} + \frac{\beta}{\bar{\rho}^2} \quad (2)$$

where α , β are constants and R_0 is the mechanical radius of gyration of an object the same shape as the particle. The plot of R^2 against $1/\bar{\rho}$ for C1q proved to be predominantly linear and, assuming this, gave an infinite contrast result for R_0 of 139.5 ± 2.5 Å. The slope α was negative rather than positive and as it is given by the second moment of the fluctuation scattering density distribution ρ_F , that is:

$$\alpha = \frac{1}{V} \int \rho_F(\mathbf{r}) r^2 d^3 \mathbf{r} \quad (3)$$

there would seem to be less than average scattering density at large distances from the centre of gravity of the molecule.

Since soluble proteins have high-scattering hydrophilic residues at their surface and low-scattering hydrophobic ones

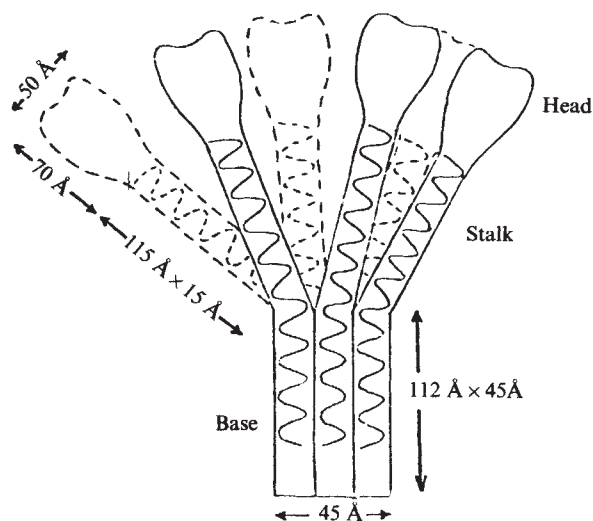


Fig. 2 Molecular structure of C1q proposed by Reid and Porter¹⁵ (after whom the figure is taken) to account for the effects of collagenase and pepsin digestion^{9,21} and the dimensions and shape of the images seen in the electron microscope⁵⁻⁷. Analysis and sequencing^{9,17,24} of the three types of polypeptide chain present show clear similarities between them, notably the repeating collagen-like triplets of residues which occupy some 85 positions starting near the N-terminal end. The characteristic Gly-X-Y pattern, where the residue Y is often either hydroxyproline or hydroxylysine, is broken at position 39 of the A chain² and 36 of the C chain¹⁷ allowing the bend of the triple helices (3) forming each stalk away from those in the base region.

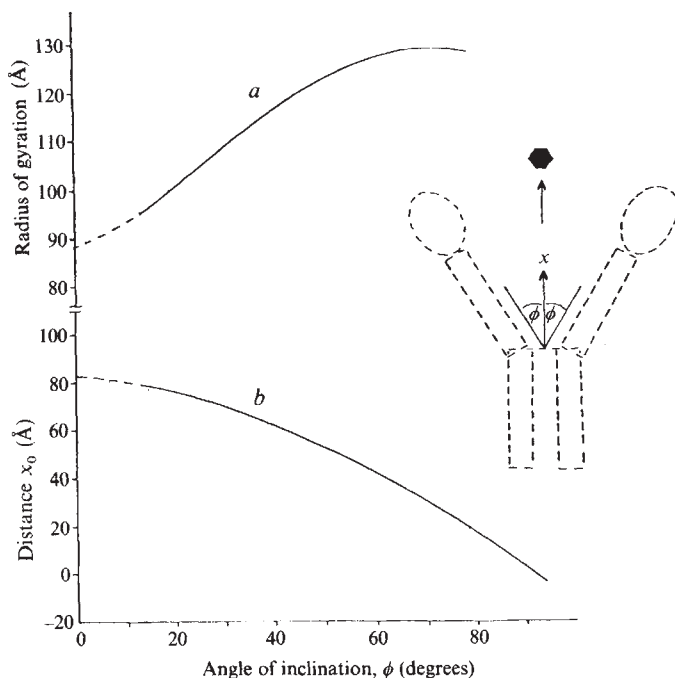


Fig. 3 Prediction of the behaviour of: *a*, the radius of gyration R_g of a uniform object the same size and shape as the C1q molecule; and *b*, the distance x_0 of its centre of gravity above the junction of base and stalks. Both the measurement of x and the angle of inclination ϕ are defined in the inset which shows a cross-section of the model. The dashed portions of each curve cannot be realised as ϕ is less than the critical angle at which the globular regions touch one another.

inside, it follows that the C1q molecule does not have a simple globular form. Biochemical and electron microscopic evidence^{5-7,15-17} has established that instead it has the appearance of a bunch of tulips (Fig. 2). Eighteen chains, each some 200 amino acid residues long, are linked in threes in the base and stalk regions to form collagen-like triple helices and each of the six fibres terminates in a globular head region. At least one head is held by each C_H2 domain when binding to the Fc region of the immunoglobulin molecule takes place^{18,19} and it has recently been suggested²⁰ that sugar chains protruding from the heads act as recognition sites.

Calculations based on the dimensions shown in Fig. 2 and assuming the 6-fold symmetry seen in recent electron micrographs (K. B. M. Reid, personal communication) show that variation of the angle of inclination ϕ of the stalks to the direction of the hexad axis has a marked effect on the resulting geometric radius of gyration R_g . Right circular cylinders of radius 7.5 Å were taken to model the stalks and also the base, where six were arranged with the centres of their ends at the vertices of a hexagon of side 15 Å and the central enclosed volume was assumed to be unoccupied. The globular regions were represented by ellipsoids of revolution with semi-axes of 25 Å, 25 Å and 35 Å. Unfortunately, no estimates are available for the size and shape of the cleft which seems to divide each head into two distinct components when viewed from above⁵. For purposes of calculation a sphere of radius 17 Å was removed from the top of each ellipsoid. This reduced both the total volume to the value determined above and the proportion of it occupied by the six heads to 55%, similar to the 57% contribution found in weight analysis²¹.

Summation of the moments of inertia of the various parts of the model about a point on the hexad axis at a height x above the junction of base and stalks and minimizing the resulting

expression gives $x (=x_0)$ corresponding to the centre of gravity for given ϕ . Returning this relationship to the equation for the total moment of inertia yields the dependence of the radius of gyration R_g on ϕ . As can be seen from Fig. 3, the 14° minimum inclination corresponding to the tulip heads in contact produces a structure with an R_g of only 95 Å, whereas the radii for angles of greater than 60° are close to the maximum of about 132 Å which occurs for $x_0=0$. Variation of the model to allow for differently shaped clefts seems to have a limited effect on the qualitative behaviour or the values obtained. The calculations were also repeated to assess the influence of errors in the known parameters. It is clear that the form of the R_g versus ϕ dependence is only significantly changed by unrealistic distortions and any proportional increase in R_g is only achieved by at least the same percentage rise in one or more dimensions of each component of the model.

As C1q is present in only very small quantities in serum and its isolation is difficult, we have not so far investigated the concentration dependence of our neutron measurements of R_g . Interparticle interference usually decreases the scattering intensities at lower Q (ref. 22) so the result of 139.5 Å would be expected to rise on extrapolation to infinite dilution. At 3 mg ml⁻¹, however, the concentration we used is already below the 5 mg ml⁻¹ recommended by Pilz²³ for negligible interference effects and, although the protein is basic ($pI \approx 10$) and therefore positively charged at pH 7.4, the ionic strength of the buffer was enhanced by the addition of 0.15 M sodium chloride. Both our own and other neutron observations of proteins indicate that unless considerable aggregation were present (which we do not believe to be the case—see legend to Fig. 1), any change in the radius of gyration would be less than 1%. Confirmatory data from solutions at additional contrasts are also desirable but two conclusions can be drawn: first, that the dry volume of the C1q molecule determined from the neutron scattering of solutions is consistent with the structural dimensions established by electron microscopic and biochemical techniques; and second, that its conformation in solution is an open one with the angle of inclination of the tulip stalks at least the 60° corresponding to the average head-to-head bunch diameter seen in the electron micrographs of Shelton *et al.*⁵. The assumption that the conformation is unique is supported by the high statistical correlation coefficients observed in the Guinier region and by the possibility that an otherwise anomalous peak seen at $Q \approx 0.037 \text{ Å}^{-1}$ in further spectra from the same specimens is a weak subsidiary maximum.

We thank Dr K. B. M. Reid for helpful discussions and both the Medical and Science Research Councils for grants to support this work.

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BOOK REVIEWS

Light on the Pauli legend

John Stachel

THIS volume is the first of a series which is to include the complete scientific correspondence of Wolfgang Pauli. Pauli played a central role in the development of quantum mechanics in the 1920s, and in its interpretation and acceptance by the physics community. Among his achievements during the period covered by this volume were his writing of the classic exposition of relativity theory as a lad of twenty; his development of the Pauli or exclusion principle, for which he later won the Nobel Prize; his proof of the mathematical equivalence of matrix and wave mechanics, worked out independently of the better-known proof by Schroedinger; and his work with Jordan and Heisenberg on the foundations of quantum field theory. Weisskopf, in his Preface, tries to characterize Pauli's approach:

Pauli had a special way of doing science. He created a unique style of thinking and research, which has deeply influenced and guided physics. It is a style that emphasizes the essential and the symmetrical in the laws of nature, and captures it in mathematical formulas without many words or empty talk. In the minds of all physicists, his mode of thinking and his whole nature stand as something ideal, clear and pure.

Something of the awe in which his intellectual powers were held may be surmised from the nickname bestowed upon him in the German-speaking physics community: 'Zweistein' (or 'two-stone'), second only to 'Einstein' (or 'one-stone').

Many stories about his formidable personality do not suggest a similar comparison with the popular saintly image of Einstein, and this volume certainly provides some documentary evidence to reinforce the Pauli legend (see the letter to Einstein quoted on p. xxxvi of the Introduction, for example), though also many examples of his helpfulness to those in difficult circumstances (see p. xxii of the Introduction, for example). In his Preface, Weisskopf gives some brief insights into Pauli's personality. On the whole, however, the annotations are rather reticent on the subject of his personal life. I could find no reference anywhere in the volume to Pauli's Jewish ancestry. The valuable chronology through 1929 on pp. 536-541 confines itself to his scientific activities and travels, avoiding such matters as the date of his marriage, with one curious exception: the date on which he

Wolfgang Pauli: Scientific Correspondence with Bohr, Einstein, Heisenberg, A.O. Volume I: 1919-1929. Edited by A. Hermann, K. von Meyenn and V.F. Weisskopf. Pp. 577. (Springer: Heidelberg and New York, 1979.) DM 160, \$88.

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Wolfgang Pauli, 1900-1958

left the Roman Catholic Church is given. Perhaps this reticence befits a critical edition of his letters, which provides indispensable material for a critical biography but cannot take the place of such a biography.

In his valuable Introduction, Professor Hermann rightly stresses the importance of such collections of scientific correspondence.

The communications system of the 'scientific community' in the twentieth century is based upon periodicals and letters. Periodicals publication is strictly regulated. Therefore the supplementary information value of letters is considerable. 'Informational value' means in the first place informational value for the recipient of the letter, therefore for contemporary physicists, but also means informational value for present-day historians of science. Historical writing which only utilizes contemporary publications can only be considered as a first beginning. Collections of letters facilitate scientific-historical work at a higher informational level.

I would add that another major source of scientific communication in this century is verbal — either the private (face-to-face

or on the telephone) or public forms (such as lectures and discussions). The former is almost always irretrievably lost, unless preserved in letters, contemporary notes or later memoirs; the latter is more often preserved in the form of conference proceedings — often altered in form by the time they reach print. Still, these are precious sources of information which should not be neglected by the historian of science.

Quotations from later letters in the course of Hermann's discussion of Pauli's role in the development of modern physics whet our appetite for future volumes. A few of Hermann's comments may raise some eyebrows, for example (p. xix): "Intellectual creation is something peculiarly human and consequently independent of time". If this were taken literally, it would be hard to see what the function of the historian of science could be.

The book proper consists of the texts of 241 letters to and from Pauli, together with running commentaries linking groups of letters and extensive annotations of the letters. Texts and commentary are in German. The letters give a fascinating picture of the interactions between Pauli and a few of the handful of physicists who were re-making theoretical physics in the 1920s. In spite of the title, only three of the letters are to (two) or from (one) Einstein; over half the letters are to or from Bohr and Heisenberg, in roughly equal number. Others with an exchange of over ten letters are Ehrenfest, Kronig and Landé. If one wants to derive the full value from their scientific content, the letters are best read in conjunction with Pauli's published papers, available in one of those facsimile collections which too often substitute for complete, critical editions of the writings of a scientist (*Collected Scientific Papers by Wolfgang Pauli*. Edited by R. Kronig and V.F. Weisskopf. Interscience: New York, 1964. 2 volumes). Fortunately, the extraordinarily rich and detailed annotations (which I understand are mainly the work of Dr von Meyenn) make reference to these papers, as well as to other contemporary writings and later historical studies, very simple. The reader will also be most grateful for the commentaries and annotations explaining the context of the letters, and various references to persons and events which otherwise would send him/her

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Pauli with his wife, Francis, in Stockholm to
receive the Nobel Prize for Physics, 1946

scurrying to the reference books.

The principles of inclusion of the material are not quite clear. In "Postscript and Indications for the User", it is claimed that all the scientific correspondence so far collected is included; yet we are told that "letters with markedly sterile content form an exception" (p. 534). The example given, letters from Felix Klein concerning the editing of Pauli's famous relativity treatise, could conceivably interest someone studying scientific institutions. Still, a list of the omitted letters can be constructed from the "Alphabetical List of Correspondence" at the end of the volume. Aside from seven Klein letters, only seven others appear to

have been omitted. In the few cases where I could compare the transcription of letters in this volume with other transcriptions or the originals, they seemed accurate. The one exception, oddly enough, occurs in the only facsimile of a Pauli letter printed in the volume, between the Preface and the text. Reference to the printed version of the letter shows that a line above the salutation has been left out. While not enough to shake my faith in the accuracy of the texts, this omission did give me pause. Incidentally, one would have liked more facsimiles and photographs. While nothing takes the place of handling the originals, a good facsimile can help the reader to

capture something of the flavour of the original.

Of more concern, perhaps, is the very concept of a division between scientific and non-scientific letters. For example, I understand that Pauli's correspondence with C.G. Jung is not to be included in the forthcoming volumes. While this may be a gratifying confirmation for some of their estimate of the scientific status of Jungian psychology, it certainly will deprive us of a great deal of insight into an important encounter for modern intellectual history, one which led to Pauli's study of Kepler and influenced his later epistemology. More generally, the division between scientific and non-scientific aspects of a life's work is not a simple matter. Certainly, modern approaches to the study of the history and philosophy of science, let alone the study of the scientific creative process which so fascinated Pauli, do not and cannot confine themselves to any narrow definition of such a distinction.

But one must not cavil. In this edition we are being given an excellently edited, annotated and indexed collection of the most important letters of a pivotal figure in the development of modern physics, which will be of interest to historians of science, physicists concerned with their traditions, and more generally all those fascinated by the creative process at its highest level. These letters will contribute immeasurably to our understanding of Pauli the man, his times and his achievement. □

John Stachel is a Professor at Boston University, currently working at the Institute for Advanced Study, Princeton, New Jersey.

Thinking of the mind

Stuart Sutherland

Mind and Nature. A Necessary Unity. By Gregory Bateson. Pp.238. (Wildwood House: London, 1979.) £7.50.

THE LAST infirmity of noble minds is the desire to create a grand synthesis of everything. Even such a down-to-earth physiologist as Sherrington succumbed by writing in his old age the impenetrable *Man on His Nature*. At the age of 76 Gregory Bateson, one of the pioneers of social anthropology, has produced his own contribution to this genre. The collocation of words in the title, *Mind and Nature. A Necessary Unity*, is sufficient warning of what to expect in the contents.

The core of Dr Bateson's book is his list of six criteria for regarding something as a mind, as follows: (1) A mind is made up of interacting parts — thought could not be carried out by an entity not composed of parts, if only because there would be no way of representing external states of

affairs within it. (2) Mental processes are triggered by differences or by changes — information can only be carried by difference. (3) Mental processes require energy generated from within the system — they are not simply reactions to an externally applied force, unlike the motion of a bullet fired from a gun. (4) They contain circular chains of causation often involving negative feedback. (5) In mental processes, the effects of a difference detected by the system are to be regarded as a coded version of the difference itself — Dr Bateson seems to mean by this that mental structures represent aspects of the external world. (6) Mental processes involve a hierarchy of logical types — this would appear to mean that one mental process can serve as the input to another and that minds are capable of reflecting on aspects of their own activity.

Although some of the criteria are obscurely expressed, most people would probably be prepared to grant that they are

necessary conditions for the existence of mind. Unfortunately, Dr Bateson does not discuss whether they are *sufficient* conditions. They are in fact all met by a computer programmed to perform an intelligent task and although one would be happy to regard such a machine as working in an analogous way to mind, there is little temptation to think of it as actually possessing a mind. Dr Bateson deliberately ignores the problem of consciousness and indeed appears to attribute mind to plants and to the evolutionary process. But the differences between the workings of the evolutionary process and those of the mind may be more interesting than the similarities. He has in fact omitted from his list of criteria for mind the one that many would regard as the most important, namely, the characteristic of acting by intention, something that clearly distinguishes the blind forces of evolution from the human mind. One way of putting this is that minds contain explicit representations of their own goals in the light of which the representation of the external world can be manipulated in order to formulate a suitable course of action. The representation of future states of

affairs is at best very indirect in plants and would appear to be non-existent in the evolutionary process.

Despite its inadequacy, Dr Bateson's discussion of the nature of mind is the clearest section of his book. The remainder discusses some of the prerequisites for learning and insight and the problem of evolution, but I found it impossible to understand the general drift of the argument let alone to summarize it. He illustrates his themes with examples drawn from many different fields: few are novel and it is unclear what conclusions he draws from them. He notes, for instance, that the equation $(a+b)^2 = a^2 + 2ab + b^2$ can be represented geometrically as a square with sides of length $a+b$ and with the interior divided into four rectangles of areas a^2 , ab , ab , and b^2 . But he stops short at explaining

why such a representation is easier for most people to grasp than the algebraic equation, and the relationship between this and other examples to his main thesis remains obscure.

In short, *Mind and Nature* is a book to be read for the incidental examples strewn through it, but anyone who wants to discover its general message will have a hard time. As Heraclitus wearily steps for very much more than the second time into yet another river, one gathers that process is important and that everything is connected to everything else — the book is indeed a grand but empty synthesis of everything. □

Stuart Sutherland is Director of the Centre for Research on Perception and Cognition at the University of Sussex, Brighton, UK.

Microelectronic technology

T.H. O'Dell

Magnetic Bubble Technology. By A.H. Eschenfelder. Pp.317. (Springer: Heidelberg and New York, 1980.) DM97, \$54.40.

AT A time when microelectronic devices based upon single crystal silicon have become part of our popular culture, it is good to see a book concerned with a microelectronics based upon the far more interesting magnetic crystals, the garnets. The information-processing potential of these devices exceeds that of their silicon predecessors by at least two orders of magnitude.

While there have been two previous books on the theoretical aspects of magnetic bubbles, Eschenfelder's is the first to go into the real technical detail of devices and, like most books of this kind, a feel for the work can be obtained from a study of the index; this is good, and calls attention to Chapter 6 which is an excellent review of the available magnetic materials of bubble technology, the garnets, hexaferrites, amorphous metal films and orthoferrites. All these materials are treated very effectively using a powerful graphical technique, pioneered by Eschenfelder, in which the coordinates are chosen such that different values of exchange constant may be represented by

sets of parallel straight lines. The unification which this technique brings about is really striking.

Chapter 6 also contains a valuable treatment of magnetic anisotropy. In magnetic bubble garnets this is induced during the growth process for the film, one of the topics in Chapter 7 which is on device fabrication and includes a good discussion of lithography giving considerable numerical detail.

Chapter 8 deals briefly with device packaging, while Chapter 9 is on applications and is not just concerned with simple memories but also describes the fascinating information-processing possibilities of the technology. This enables data to be sorted as it is in the process of being stored or retrieved by means of the flow-steering switches pioneered by Eschenfelder's IBM colleagues Chen, Chang and Tung. The book concludes with Chapter 10 which takes a brief look at what the future may hold for this exciting and highly developed microelectronic technology.

The first five chapters of this book are not so good, however. No Appendix A, promised on p.1 to relate the Gaussian units, in which the book is unfortunately written, to SI units, exists. A conversion table is printed inside the front cover to rectify this omission. Figures 1.8 and 1.10 are repeated later as Figs 4.31 and 8.3. Page 8 sees the start of half a page of text with the most peculiar absence of capitals and punctuation.

Chapters 1-5 deal with bubble statics, dynamics and propagation structures, but give no help at points where earlier publications may have been obscure. An exception is to be found upon pp.156-158, where the elegant cross-hatch current sheet device due to Voegeli, and unpublished until now, is described. □

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Fungal ecology

John Webster

The Ecology of Fungi. By W. Bridge Cooke. Pp.274. (CRC Press: Boca Raton, Florida/Blackwell Scientific: Oxford, 1980.) \$77.95, £50.75.

ABOUT 100,000 species of fungi are known. They are ubiquitous in soil, in water, on organic matter of all kinds, and occur as parasites of plants and animals, and as symbionts in association with algae in lichens, and with roots forming mycorrhizas. Their prolific reproduction and effective dispersal ensure that suitable substrata are quickly colonized, and it is uncanny how, at the right place and time, on the appropriate substratum, a particular fungus can be found, indicating precise adaptation to its niche. Fungi play important roles in ecosystems, especially as parasites and as decomposers. Some — for example, lichens, or *Rhytisma acerinum*, the cause of tar-spot of sycamore — are valuable indicators of SO₂ pollution.

To review the ecology of such a group of organisms is a daunting task and, for a single author, well-nigh impossible unless he attempts severe selection of the wealth of available material. A glance at the contents page of *The Ecology of Fungi* shows that this sets out an impressive synopsis of subject matter. Unfortunately the actual text is a great disappointment. "Basically, this book is an elaboration of an earlier review of the subject . . ." (by W. B. Cooke in 1958). It is emphatically not a book for a beginner. Nowhere is there an attempt to set out the principles of the subject and many terms are introduced without definition. Most of the sections are annotated literature abstracts, such as might be made by compiling and arranging a collection of *Biological Abstracts*. Rarely is there any critical appraisal or synthesis. Too often, no conclusions are drawn, but merely a statement is made that a certain study has carried out. It is, of course, dangerous to make selected quotations out of context, but the following are offered, without the reference numbers, to give an impression of flavour.

It has been shown that collections of *Schizophyllum commune* can produce turgid spores after more than a year of complete desiccation. Assuming that a turgid spore is a viable one, spores of this species can withstand considerable desiccation.

There is an obvious confusion here. The reference is to Buller (1909), and it should have been added that the *fruit-bodies* which Buller freeze-dried have been revived some 52 years later (Ainsworth in *Nature* 195, 1120; 1965).

Transpiration was found to be a vital function in the fruit-bodies of agarics. It occurred under conditions of complete

● *Statistical Mechanics* by R. H. Fowler has been reissued in paperback by Cambridge University Press, price £18.

● *Sociobiology: The Abridged Edition* by Edward O. Wilson with drawings by Sarah Landry (pp. 366; price: hardback \$18.50, £11.10; paperback \$9.95, £5.95) has just been published by Harvard University Press.

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saturation, although at a reduced rate. The rates of transpiration and translocation were similar. The behavior of fairy rings appears to bear out the theory that transpiration and translocation are closely connected.

At least one of the cellulolytic fungi, *Memnoniella echinata*, has been studied intensively, and it has been shown that spores of this fungus and of *Stachybotrys atra*, another cellulolytic fungus, may be produced on the same mycelium.

There are also, unfortunately, some serious omissions.

To date, attempts which have been made to study the succession of development of these fungi [which inhabit dung] have been weak except for the little attention which has been given to the matter in certain laboratory exercises.

There is no mention of the work of Harper, Richardson, Wicklow, Larsen, Ikediugwu, or even the reviews by Webster or Lodha.

Few studies have been made on the succession of fungi involved [in wood decay] or their contribution to the rate of decay.

The important phenomenon of fun-

gustasis and Lockwood's review (*Biol. Rev.* 52; 1977) are completely ignored. There is no mention of heterogenic incompatibility. The effects of C/N ratio on fungus growth are covered in four lines and one reference. The subject of mating behaviour in *Achlya* is discussed without reference to antheridiol and oogoniol, and that of the Mucorales without reference to trisporic acid. The illustrations are sparse, consisting mainly of graphs or histograms, with relatively few, badly reproduced half-tones. The number of misprints is high.

The most useful feature of the book is the list of 1293 references. But, as already indicated, it is seriously lacking in some subject areas, and is not very recent (116 references post-1975, 19 references dated 1977 and 1 for 1978).

With all these defects and a high price the book is not worth buying by individuals, although some well-endowed libraries may find a place for it. □

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Highways and byways of cosmology

M.J. Laird

Theoretical Cosmology. By A.K. Raychaudhuri. Pp.298. (Clarendon/Oxford University Press: Oxford, 1979.) £10.

IN THE preface, the author expresses the hope that this book will prove both a handy reference for the professional cosmologist and a readable and informative review for the advanced graduate student and the physicist who is not a cosmologist. I should state straightaway that I am numbered among the non-cosmologists, though I was supervised by one cosmologist (H. Bondi) and did post-doctoral work under another (T. Gold).

Now for the contents of the book. After a brief introduction, the author presents the standard Newtonian and relativistic cosmologies. There are chapters on the microwave radiation background, thermal history and nucleosynthesis, singularities, perturbations, galaxy formation, and on the tricky business of analysis and interpretation of observational data. However, a particular feature is the inclusion of a wide range of models and theories. Some of these, such as relativistic models not satisfying the cosmological principle, varying gravitational constant and Brans-Dicke theory, and Einstein-Cartan theory, are considered in some detail. Others — two-

tensor theory, the conformally invariant theory of Hoyle and Narlikar, and theories involving equal amounts of matter and antimatter — receive brief mentions. At the end of the book, the author discusses the extragalactic radio sources, and those hardy annuals, Mach's principle and Olbers' paradox.

The book is reproduced from typescript. There are some errors, including the definition of the Ricci tensor, and some places where one could be confused. For example, on one page *R* appears in the field equations, and on the next as the scale factor in the Robertson-Walker metric without indication that the meaning has changed. The author also writes "deuteron" for both deuterium and the deuterium nucleus.

The exposition is certainly that of the review rather than that of the monograph or treatise. Many results are presented rather than derived, though there are many references (about 800). I can vividly remember meetings of the Royal Astronomical Society at which cosmologists spoke with evangelical fervour. In contrast, the author takes a rather dispassionate view. As far as the stated objectives are concerned, I think that the non-cosmologist with no prior knowledge of the subject, or at least of general relativity, would find parts of the book fairly hard going. The cosmologist (or prospective cosmologist) might find it a useful guide to the highways and a reminder of (or introduction to) what are currently some of the byways of cosmological theory. □

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One lake in great depth

Brian Moss

Neusiedlersee. Edited by H. Löffler. Pp.559. (W. Junk: The Hague, 1980.) DG195, \$102.65.

SOME limnologists find their souls in the immensity of the deep glacial lakes of the Alps and Norway, others in the heat-trembling far shore of a Rift Valley salt lake. And some join Wilfred Thesiger and Majorie Stoneman Douglas in their feeling for the great marshes, where, for a time, the tall reeds still shut out the abuses, often sanctioned as acceptable water quality, now heaped on the world's fresh water.

The Neusiedlersee, a shallow, slightly saline lake linking Austria and Hungary, is half occupied by a reedswamp the beauty of which is celebrated in some colour plates at the end of this book. Like other such shallow lakes it has a fascinating history of co-existence with local peoples who exploited the reeds, sedges, fishes and wildfowl without upsetting the intricate relationships supporting the ecosystem itself. More pressure is now placed on the lake — eutrophication, tourism, tamperings with the water level — but it has fortunately yet suffered less than some comparable water bodies and hence has been an ideal subject for the many-sided investigation which is the subject of this book.

There are 30 chapters, some lengthy and of wide scope ("The Catchment Area, a Geographical Review" for example), others extremely short — one of a single page — or highly specialist (for instance on the distribution of a single species of ostracod). They contain an immense amount of information and a close reading produces as full a picture of the limnology and biota as might be expected given the man-power and financial limitations of all such studies. Particularly full are the sections on aquatic plants and birds; invertebrates are well covered as are algae from a floristic point of view, and the geological and physical information is detailed. Data on the more important inorganic nutrients are scarce. Scientists working on shallow lakes, brackish waters and swamps will find useful comparative information and the impression given is of thoroughness and precision in the listing of details.

Neusiedlersee is, however, very much a collection of separate pieces of work. Professor Löffler, the editor, has laboured hard to bring together the data of over 20 contributors, but has not seen fit to write or commission a chapter which attempts a synthesis. The orthodox separation of those who work on the open-water zooplankton or the productivity of submerged plants is hallowed. It is not easy

to see how the immense production, largely ungrazed, of the reed zone may influence the organisms of the open water through, for example, export as detritus or through the cover and spawning needs of open-water fish. I am sure the authors have some sort of mental model of the integration of these and other processes and regret that they have not given some inkling of it. Projection and speculation are of equal interest to other workers as are established facts and not necessarily any less valuable if the facts are given.

The book costs no mean sum, even if book prices have not risen, relative to salaries, as rapidly as rumour would have it. It is solidly produced but will have a

fairly narrow market, so I suppose the fairest way of deciding whether the £44 or so is justified is to consider the cost of obtaining the information through Inter-Library Loan were it scattered in journals. With nearly 500 pages of text, exclusive of species and general indexes and colour plates, I should think that the effort of making out all the necessary request cards plus the more obvious costs of the loan system would justify the buying of the book by libraries and by anyone closely involved with the field who has repaid his or her mortgage! □

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A change of climate

Graham Farmer and Jean Palutikof

Weather Force. By John Gribbin. Pp.188. (Hamlyn: London and New York, 1979.) £6.95. *Causes of Climate*. By J.G. Lockwood. Pp.260. (Edward Arnold: London, 1979.) Hardback £12.95; paperback £5.95. *World Climate*. By T.F. Gaskell and M. Morris. Pp. 144. (Thames and Hudson: London, 1979.) £7.95.

INTEREST in climatic change has grown in recent years to span almost the entire spectrum of society. The interest is in response to a number of factors: growing awareness of Man's capacity to modify the weather and climate; the recent experience of extreme conditions such as the UK drought of 1976 and the severe winter of 1978–1979; finally, perhaps, a realization that since global resources barely fulfil the demands made upon them today, any change in climate could have catastrophic consequences.

Inevitably, out of this general interest has sprung a new breed of introductory books on climatology, devoting a large proportion of the text to the subject of climatic change. This bias is shared by the three books reviewed here. And because of it, the books have in common a further structural element. To make room for the extensive treatment of climatic change, the traditional introduction to climatology through an explanation of elementary atmospheric physics is at best only cursorily treated.

The books differ greatly in level. Whereas Gribbin's book is clearly designed for the interested layman, and provides very good value for money at that level, Lockwood has produced a text for university and college introductory courses in climatology. The book by Gaskell and Morris falls between these two extremes, and as a result suffers from a problem common to all attempts at compromise, uncertainty.

Weather Force, by John Gribbin, does not set out to teach meteorology or climatology, but looks instead at the impact of climate and specific weather phenomena on Man and society. This is done through a somewhat sensationalist, but very well illustrated (there are some 200 illustrations, mostly photographs, in the 190-page text), account of extreme weather events such as hurricanes, drought, floods and record-breaking hailstones. There is heavy reliance on visual impact, with lavish photographs and items of note blocked off into boxes. The latter technique does lead to some repetition and awkwardness in following the main text.

Both Chapters 1 and 3 are devoted to the discussion of extreme weather events. These cover not only the severe winters and droughts of the 1970s, but go back in time to the Solway Firth bogburst of 1771 and beyond. Flooding and hurricanes have the greatest coverage, enlivened by numerous personal accounts. One problem, especially in Chapter 1, is that Gribbin seldom gives more than one side of an argument. For example, it is stated as fact that both the severity and the frequency of extreme events increased during the last decade, and that this trend will persist in the future. No mention is made of the extensive statistical work, such as the well-known study by Ratcliffe *et al.* (*Q.J.R. met Soc.* 104, 243–255; 1978) which shows no such increase in variability.

Chapter 2 is a general summary of climatology. As such it is perhaps somewhat misplaced, but to have begun the book with such a summary would have led to loss of impact. It is unfortunate that some of the diagrams here are not up to the book's general high standard. For example, in the representation of a warm front the frontal line in the air does not meet the frontal line on the ground, and the diagram showing states of water in the atmosphere is confusing and lacks sufficient contrast.

Climatic change, the cause of changes in the frequency of occurrence of extreme events, is discussed in Chapters 4 and 5. There are some questionable points

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Frost Fair on the Thames, 1683–1684

regarding, for example, Scottish policy in the seventeenth and eighteenth centuries. The theory presented is disputed by many historians, but Gribbin again fails to present both sides of the argument. There are also contradictions, such as the statement that a new Ice Age would cause the Sahara to be "as bad as ever" (p.47), yet would provide "rich supplies of grain, meat and milk" (p.95).

Chapter 6, "Climate and Food", is an excellent introduction to the political and economic implications of food shortages in the developing world. Gribbin presents a well-balanced argument to show that enough food is available today to feed the world's population, were it not for human factors. The same theme of climate and Man is continued in the final chapter looking at weather modification, with an unusual but interesting section, "Who pays when things go wrong?"

In general, this is a professionally written and entertaining book. It would have been improved by a list of further reading beyond the two books (both by John Gribbin) mentioned in the text. There is also some danger in the one-sided presentation of views, sometimes against current scientific opinion.

John Lockwood's book, *Causes of Climate*, is intended, according to the Preface, "to provide arts and social science students with an introduction to modern climatology, and science students with a basis for further advanced study". For the non-science reader, this book assumes too much knowledge. A lecturer using it as a basic text would, for example, have to tread very carefully through the development of the Bowen ratio on pp.56–57. Terms such as specific humidity and thermal inertia are used with scant explanation. For the scientist this approach is highly satisfactory: it avoids the often laboured explanations of other standard texts trying to accommodate scientists and non-scientists alike.

Another positive attribute of *Causes of Climate* is that the standard approach of

laying a groundwork of meteorology separate from and before introducing any climatology is not used. Instead, the two are carefully woven together with the emphasis on climatology, drawing in meteorology only as and when it is necessary to elucidate a point.

The introduction is made through the general concept of systems, leading up to the climate system and the role of energy within that system. Radiation is discussed in detail and at length in the second chapter, as the prime energy source of the atmosphere. The following two chapters cover the nature of surface climates, first through the role of different types of surfaces, and second through atmospheric circulation patterns. More should have been included at this point on water in the atmosphere and in atmospheric systems such as fronts. In general, the book is inadequate in this respect.

The last two chapters are perhaps the least satisfactory. Some 12 pages in Chapter 6 entitled "The Climate Future: Climatic Models and Trends" are devoted to a detailed explanation of a hydrological model for the central Pennines devised by Lockwood and Venkatasawmy (*J. Hydrol.* 26, 79–94; 1975). This appears to be largely irrelevant to the flow of reasoning in the chapter, and improvement would result from either discarding it or replacing it by a more appropriate example. Chapter 7, "Man and Climate", is only seven pages long, and deals with alternative climatic scenarios in the next century and their impact. Given that climate has a crucial economic role in this over-populated and under-fed world, this chapter could well have been expanded.

Overall, Lockwood's book is an excellent addition to the range of basic climatology texts, particularly for those seeking a new approach to the discipline. Although there are some omissions, these are of topics more than adequately covered elsewhere, and this is compensated for by the wealth of new material provided.

World Climate by Gaskell and Morris

attempts to approach its subject at a level somewhere between the other two books. The result is unsuccessful. The text abounds with statements which are at best unhelpful and at worst misleading: "Although there is not much air around in the stratosphere . . ." (p.100), "Part of the sun's energy is . . . trapped in the space above the atmosphere" (p.99). The book carries with it an air of haste which manifests itself in major and minor inaccuracies throughout.

After a short opening chapter, "Facts and Fluctuations", the authors attempt to condense into 12 pages the contents of an introductory meteorology textbook. For the reader this must lead to confusion, further reinforced by inaccuracy. For example, centrifugal force is mentioned in the text but not incorporated in the accompanying diagram (p.16), so that straight-line rather than curved flow would result from the balance of forces shown.

The straightforward chapters on meteorology and weather forecasting are well written and clear. The use of the six-season approach to describe the annual trend of atmospheric circulation is a useful technique. Chapter 7, "Heat from the Sun", would have been better placed amongst the earlier chapters on introductory meteorology.

Special mention must be made of the diagrams. It is disconcerting to see that acknowledgements are not given for figures taken from other works. Figure 27, showing the Russian rivers scheme, is taken without acknowledgement from p.183 of the out-of-date book by Overman (*Water: Solutions to a Problem of Supply and Demand*. Aldus: London, 1968). Figures 16 and 17 are taken from p.432 of Lamb (*Climatic History and the Future*. Methuen: London, 1977), but the caption of Gaskell and Morris is incorrect. The map is from a sixteenth (not fourteenth) century manuscript, and is drawn with a much higher degree of imaginative interpolation than Gaskell and Morris suggest. To compound these inadequacies, some of the other figures are of little use and contain inaccuracies. For example, the stylized radiation balance bears little resemblance to the correct, more intricate pattern and the caption is misleading.

The overall confusion which abounds in this book is symbolized in an example from the index: a reader searching for information on "oxygen isotopes", "cores" or "Emiliani" is directed to a photograph (p.65) of a glacial valley in Merionethshire.

Gribbin and Lockwood are to be congratulated on producing two fine and complementary books. They are recommended to all, including Gaskell and Morris. □

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